Characterisation of the biological potential of fracture non-union tissue

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Abstract

Over 1.1million fractures are estimated to occur annually in England and Wales. Up to 10% of these are likely be complicated by a delay or failure to heal with significant health and financial implications. Definitions of fracture non-union are not clear and although the Weber and Cech classification is still the definitive, misinterpretations remain common. Current treatment is surgical but the morbidity, particularly where autologous bone grafting is used, can be as high as 30%. Novel approaches are being tried but few of the strategies have made the translational impact that the laboratory and animal model data suggested.

The work presented here investigates the feasibility of quantifying the biological potential of non-unions in patients and in a validated, *in vivo* model.

Hypothesis: there is quantifiable biological potential in fracture non-union tissue that can be stimulated leading to osseous union by closed percutaneous injection of induction factors.

Tissue from patients with non-infected fracture non-union undergoing treatment was examined to determine the feasibility of quantifying gene activity in small samples of non-union gap tissue.

Non-union tissue from the animal model of an established non-union was examined to assess its osteoblastic potential by culture of extracted cells.

Orthobiological agents that have shown great potential in gap models of non-union (BMP2 delivered in a viral construct (AdBMP2) and the thrombin peptide, TP508) were assessed to determine their efficacy in a clinically analogous model of fracture non-union.

Quantifiable metabolic activity was found in the small samples of human non-union tissue. There was potential to correlate this to the histomorphometric architecture of the tissue.

Cells extracted from the gap tissue of a non-union site in the rat model demonstrated osteoblastic potential *in vitro*. However, percutaneous injection of the orthobiological agents into the non-union site *in vivo* failed to stimulate healing.

The tissue at the site of a fracture non-union has a quantifiable metabolic activity that may have great clinical application and research benefits. Tissue from the non-union site of the animal model did demonstrate osteoblastic capacity but attempts to effect healing using percutaneously injected orthobiological agents that have previously shown potential, failed. This may be due to the chronic timepoint chosen to replicate the clinical situation. Further work is necessary to determine the prognostic potential of the gene assays and to continue to characterise the biological potential of the non-union tissue so that interventions can be more accurately directed.

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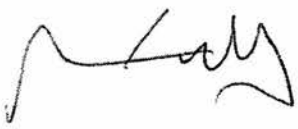
uthor's declaration

declare that the work in this thesis was composed by the author and that it has not been ubmitted for any other degree or professional qualification. Except where indicated by pecific reference in the text, the work is the candidate's own work. Work done in ollaboration with or with the assistance of others is indicated as such. Any views expresses the discussion are those of the author.

gned:  **Dated:** 21st May 2012

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Chapter 1: Classification and treatment of fracture non-union

1.1 The implications of fracture non-union

Over 1.1million fractures are estimated to occur annually in England and Wales[1] and of these up to 10% are likely to have problems with a delay or failure to heal[2]. The potential burden to healthcare is enormous, estimated as almost £14000 for each non-union[3]. In addition there is the societal cost in terms of loss of earnings and prolonged incapacity that has never been quantified. Currently surgical intervention remains the standard but significant morbidity can occur in up to 30% of cases[4]. Given this, there continues to be a major drive to develop better strategies with considerable industry interest[3, 5-7].

1.2 Definition of fracture non-union

Attempts to address the problems of fracture non-union are not new. Hitzrot[8] reviewed the problem in 1926 and unfortunately, there remain many problems, not least the inability to agree a definition[9]. Without consistency of definition in the literature quantification of the problem and stratification of treatments is almost impossible[10]. Reported rates for non-union vary from 0-15% for the humerus, 0-12% for the femur[11] and from 0-80% in studies reporting on the different presentations of tibial fractures[12], so this task is not straightforward. Fracture healing is described using a combination of clinical and radiographic findings[9]. Clinically, the absence of pain and return of functional ability indicates successful healing.

Radiographically, this is indicated by progression of the ossification process and commonly the number of cortices that have 'bridging' callus, i.e. new bone crossing the fracture gap. Bhandari *et al*[10] sent questionnaires to the 577 practising orthopaedic surgeons. They had a response rate of 77% and found no consensus in the definition of fracture healing, delayed union (1-8months) or non-union (2-12 months). For the purpose of licensing treatments and devices, the Food and Drugs Administration in the United States defined this as a minimum of nine months since fracture with no visible evidence of healing for three months [13]. There are practical problems with this, not least waiting for nine months with young or active patients before commencing treatment. Frolke *et al* [14] took a more pragmatic approach, defining fracture non-union as a disturbance of normal fracture healing with the expectation that no consolidation will be achieved without focused treatment. An alternative view considers that six rather than 9 months should have elapsed with no clinical or radiological evidence of progression before it is termed a non-union rather than a delayed union. There does not appear to be any published evidence for 6 months but it is commonly used[15].

1.3 Classification of fracture non union

Weber and Cech's morphological system, reported in 1976[16] is still the most widely used classification. Other attempts have been made to further classify the clinical picture. McKee proposed a system in the AO principles text[17]. It derives from that of Weber and Cech but excludes their description of the specific characteristics of the atrophic type. Another example is that proposed by Paley for tibias[18] based on the Ilizarov school. Again it appears to be an adaption of that of Weber and Cech. Megas[19] found few classifications in their review, suggesting the common theme that they were either atrophic or hypertrophic. Thier description seems to be an simplification of the Weber and Cech work. Interestingly this now appears to be the accepted standard as demonstrated by Marino and Ziran[20]. They reproduced the Weber and Cech diagram depicting types of pseudoarthroses but naming Weber and Cech's 'horse's hoof' and oligotrophic non-unions as oligotrophic and atrophic respectively, in effect shifting the paradigm without reference or explanation.

The system of Weber and Cech therefore remains the most comprehensive and accurate. It was derived from a combined experience of approximately 700 cases treated in either St. Galen, Switzerland or in Prague, Czech. It divided fracture non-union into 'pseudarthroses' that have the potential to heal and those that do not. These are commonly referred to as hypertrophic and atrophic non-unions respectively. Weber and Cech quantified the vascularity of the non-unions (pseudoarthroses) using scintigraphy and equated this to the biological potential. In their original diagram the legend comments that the oligotrophic pseudoarthrosis 'is mistakenly included amongst the so-called atrophic pseudarthroses'. This indeed continues to be the case. Their group of atrophic non-unions ('defect pseudarthroses') are now referred to as gap defects. Therefore according to their system, most of those fractures that are now classified as atrophic non-unions retain the potential to heal if, according to their conclusions, the mechanical environment can be addressed. They went on to conclude from their experience that the atrophic pseudorthrosis often quoted in the literature is only rarely inactive in the biological sense. Despite this, that inaccuracy has persisted and groups continue to recommend tumour-like excision of what would have originally been classified oligotrophic non-unions[21].

1.4 Factors associated with aseptic fracture non-union

This work has focused on aseptic non-unions. Infection is a very ominous factor associated with impairment of fracture healing. It is a complex process, the treatment of which is difficult and protracted[22].

There are many clinical factors associated with problematic fracture healing and non-union (Table 1). Gaston[2] reviewed the evidence for these patient factors implicated and found many of the clinical papers to be based on opinion or retrospective cohort. Much of the opinion is based on animal work or *in vitro* studies[23-25].

Table 1 Host characteristics that negatively affect bone healing

Age	Gruber[26], Robinson[27], Parker[28]
Peripheral vascular disease	Brinker[29], Dickson[29]
Malnutrition	Dwyer[30], Einhorn[31]
Anaemia	Heppenstall[32], Rothman[33]
Diabetes Mellitis	Cozen[34], Gandhi[35], Loder[36]
Endocrine	Brinker[37], Ohlsson[38], Olney[39]
Drugs (NSAIDS, Steroids)	Gerstenfeld[24, 40-41], Simon[23], Giannoudis[42] Waters[43], Goodman[44], Kagel[45], Pountos[45]
Smoking	Adams[46], Castillo[47], Gullihorn[25], Nasell[48]

Fracture and treatment characteristics have also been reported as contributing to the risk of non-union[12]. Calori *et al*[49] proposed a new scoring system based largely on their experience that combined the clinical factors with fracture characteristics and treatment strategies as a means of predicting union. It is comprehensive and although it has been reported as being accurate in one retrospective series[50], it has not been widely embraced.

Table 2 Fracture characteristics associated with delayed/non-union (Bhandari[51])

Open fracture
Transverse fracture
Post op fracture gap

Bhandari *et al* [51] studied both the clinical factors and the fracture characteristics associated with delayed and non-union in tibial fractures in 200 patients treated at two university centres. They found three simple and robust factors predictive of re-operation (i.e. for delayed or failure to heal). These were open fracture, transverse fracture and post-operative fracture gap (Table 2).

In many respects these data support the proposal of Weber and Cech in 1976 that the factors affecting the potential of a fracture to unite are either biological or mechanical. There can be overlap, for example, a fracture gap that is too large will adversely affect both the mechanics and the biology of the healing process but in the majority of cases it appears that the mechanical factors that predominate.

1.5 Diagnosis of fracture non-union

The diagnosis of fracture non-union presents a major problem. Bhandari *et al*[10] found no consensus amongst 444 surgeons replying to their questionnaire. A combination of clinical and plain radiographic findings remain the standard but functional pain can be a feature even after successful union[52-53] and plain radiographs can be difficult to interpret[54]. Other methods have been tried such as bending stiffness[55-56] and labelled scans[57] but none have become part of clinical practice. Plain radiographs remain the main diagnostic tool and are often combined with computerised tomography (CT) [58] as a means of confirming the diagnosis and planning treatment. CT has been shown to be very sensitive but it has also been shown to have a low specificity (62%) when used in isolation, risking unnecessary procedures[59]. Other modalities (for example, MRI, SPECT) are less commonly used in long bones[60].

Further work-up of a non-union also excludes infection as the treatment of septic and aseptic non-union is quite different[22].

1.6 Treatment of aseptic fracture non-union

The current, accepted standard for treatment of fracture non-union is autologous bone grafting with or without additional or revision of the fracture fixation[6-7, 61]. The surgical intervention will vary depending on the site, the fracture characteristics and the local

expertise. The adequacy of the initial and any subsequent fixation determine the need and techniques available for the further stabilisation[51].

1.6.1 Autologous bone grafting

This involves harvesting bone from another site in the same patient. The donor bone has been defined as having three advantageous properties[62]. It contains cells that survive the transplant process and are capable of osteogenic activity. It also contains factors that induce these cells and those locally to differentiate along osteoblastic lines (osteoinduction). Finally, it provides a scaffold from which and through which bone formation can occur (osteoconduction). Another advantage to the patient is that it does not have the infection risk associated with allograft tissue. Although these techniques date back to Egyptian times [20] questions regarding indications and timing remain unanswered.

Weber and Cech[63] reserved their autologous bone grafting techniques principally for bone defects, either from the initial trauma or the debridement following infection and this approach is making a resurgence (personal communication with Professor C. Moran, Nottingham). They emphasised the importance of the recipient bed for the graft (i.e. that it was clean and well vascularised) and the need for stable osteosynthesis.

The iliac crest remains the most utilised source of autologous bone graft and harvesting techniques remain similar to those depicted in the Weber and Cech monograph. These include morsellised croutons of cancellous bone, cortical or cancellous strips and block grafts containing structurally useful cortical surfaces and cancellous bone. The reported morbidity associated with autologous bone grafting, mainly from iliac crest donor sites, suggests that up to 10% incidence of major (deep infection, further operative intervention required, prolonged pain not controlled with simple analgesics) and 40% incidence of minor complications (haematoma, wound irritation, pain controlled on simple analgesics)[64]. In addition, the cost is not negligible but often unreported. Figures are difficult to find but it has been estimated to be as high as £2413.3[65]. It should be noted that this paper, although very comprehensive in its costing of the procedure, was from a group also proposing 'polytherapy'[21], an approach that uses expensive recombinant protein. The paper formed a basis for their justification of this additional cost.

Alternatives to standard autologous bone grafting techniques have been sought to avoid the complications associated with iliac crest donor sites but all confer some surgical morbidity and until recently only limited graft availability. The reamer irrigator aspirator (Synthes, US) however is a relatively new technique that can produce comparable amounts of graft to the

iliac crest[66]. The aspirate is highly osteogenic and osteoinductive and retains osteoconductive potential[67] but although lower, there is still a risk of surgical morbidity remains[66]. In addition, it has a consistency of fine sand which can be more difficult to retain at the fracture site than cancellous bone graft and has no intrinsic mechanically stabilising properties.

1.6.2 Allograft

Allograft is an alternative to autograft that is readily available in the required quantity and confers no additional morbidity to the patient, apart from the almost negligible infective risk from the transplanted tissues. It can be used in morsellised form to augment a fracture or simply to fill defects or can be used to confer a structural advantage (for example, strut grafts). The quality of the graft can vary between specimens and there is an associated cost that can be from £600 to over £2000. The processing of the bone required to reduce the infection risk means that the properties of the material are diminished: it remains osteoconductive but the osteoinductive capacity is diminished and the osteogenic potential is no longer present.

1.6.3 BMPs and fracture non-union

Attempts have been made to improve the characteristics of allograft bone by augmenting it with substances to improve its osteoinductive capacity. This involves the addition of a recombinant protein with osteoinductive properties and bone morphogenetic proteins 2 and 7 (BMP2, BMP7) are now commonly used in clinical practice.

Bone morphogenetic proteins (BMPs) are part of the transforming growth factor β (TGFβ) family and have been shown to have bone inductive properties. The term was first coined by Marshall Urist in 1967[68-69]. BMPs are unique in that they induce terminal differentiation along osteoblastic pathways and also enhance the differentiated osteoblast functions[70]. They achieve this by binding transmembrane serine/threonine kinase receptors leading to activation of the mothers against decapentaplegic (SMAD) 1 and 5 pathways[71]. Their actions are subject to multiple regulatory steps at the extra- and intracellular level. They are unique among the osteogenic factors in that they can induce bone formation when injected in isolation into muscle or subcutaneous pouches. BMP2 and BMP7 recombinant proteins are licensed for clinical use and have been used to wither in isolation or as an augment to allograft bone with some success. Jones *et al*[72] compared allograft with recombinant BMP2 to autograft in 30 patients. They found it to be safe and as effective as autograft and it avoided the additional surgical morbidity. Friedlaender *et al*[6] used BMP 7 in tibial non-unions. They randomised 124 patients to either autograft or BMP7 and found the latter to be as effective but with less

surgical morbidity. Therefore BMPs appear to have a role but they remain very expensive (£2063-£3898 per case (local pharmacy figures)) and as yet have not been shown to be superior to autograft. They have a dose dependent effect that is independent of the host but require concentrations in excess of one thousand times higher than the body level of BMPs. Given their efficacy, several groups recommend their use as a standard in the treatment of fracture non-unions, particularly those classified as atrophic[21, 73-74] and suggested that despite the additional expense they are cost-effective[3, 65].Garrison in the Cochrane review[75] commented that the role of BMPs in fracture non-union remained unclear and that there was considerable industry involvement in the available evidence.

1.6.4 Recombinant proteins and gene therapy in fracture non-union

Other proteins that have been trialled but have made less clinical impact than BMP2 and BMP7. The thrombin peptide, TP508[76-81] has been used in clinical trials including osteoporotic wrist fractures[82]. Thrombin is a serine protease with a key role in haemostasis. Activated thrombin becomes bound within the clot it induces then is released slowly with its breakdown peptides that are themselves also biologically active. Both it and its degradation peptides promote osteoblastic differentiation and proliferation from mesenchymal progenitor cells [83] and inhibit apoptosis[84]. TP508 (Chrysalin®) is a synthetic 23 amino acid, non-proteolytic peptide representing a binding domain of human thrombin that has been shown to recruit inflammatory [85], angiogenic and mesenchymal cells and lead to increased collagen III production[86-87]. TP508 has been shown to stimulate angiogenesis in wounds [88] [76]and promote migration and proliferation of osteoblast progenitor cells [79]. A single injection of TP508 solution has also been shown to result in increased callus formation in a closed fracture model [77]and to improve ossification in models of distraction osteogenesis[76, 81, 89]. The promotion of angiogenesis combined with mesenchymal cell migration and proliferation suggested that this peptide also had potential in the treatment of atrophic non union.

Other proteins have shown considerable potential in lab or animal studies (vascular endothelial growth factor (VEGF), platelet derived growth factors (PDGF)[90-95]) but have yet to make to the translational stage.

Expense, dose and delivery systems are additional considerations when using any recombinant protein. BMP2 and BMP7 are available in powder form or as soaked in a collagen sponge to slow the elution and prolong the effect. In solution they dissipate quickly from the site of application and although carrier systems can control elution they introduce foreign material. It has been used in a collagen carrier in open tibial fractures[96]. TP508 is

available in power or microsphere form, the latter having been shown to be more efficacous[78, 97] in bone, again because of a more prolonged dissipation.

Viral vectors for gene delivery have been tried as a means of administering bioactive peptides including BMP2 [98]. The advantage is that they are much cheaper than recombinant proteins and can lead to a more sustained release that can be titrated by varying the dose [99] and virus type[100]. They involve the insertion of the gene sequence of the protein of choice as a plasmid into a replication deficient virus that is expanded on specific cell lines that provide the relevant replication exon. They are then purified for injection into the recipient tissue where the protein is produced as part of the replication cycle. Adenoviral delivered BMP2 (Ad-BMP2) has been used successfully in several gap models including rat[101]. In these models the viral construct was injected at 5-7 days following the creation of the gap. There have been a few Phase I clinical trials of gene therapy[102]. These have used adenovirus and adeno-associated virus but these vectors have limitations for use in humans because of innate immunity to the virus. Although relatively safe there are safety concerns and there has been a reported death from and adenoviral vector, albeit one not used for musculoskeletal purposes and in a severely immunocompromised host[103]. More potent constructs such as lentivirus are available[99] but pose greater safety concerns that have so far limited their use and these concerns have limited the progression of translational work[98].

Viral vectors, in addition to being powerful delivery tools have brought interesting insights into the characteristics of fracture non-union sites. These behave as a 'privileged' system that is relatively self-contained (i.e. little viral product is found in the surrounding tissues or systemically) and enjoy a degree of freedom from systemic immunosurveillance (the virus continues to produce the protein for much longer than would be anticipated in an immunocompetent host if given systemically)[104].

1.6.5 Timing of intervention

Blick and co authors [105] reviewed approaches to bone grafting in tibial fractures in the introduction to their study. Suggested time had been 6 and 12 weeks base mainly on individual series[106-108] or on texts and reports from meetings (see references Blick paper[105]). The rationale was either to alter the natural history of the anticipated delayed or non-union with grafting at 6 or 12 weeks. In their study, Blick et al reviewed those grafted within 16 weeks of the index procedure. It compared this mixed group of high energy injuries with case matched historical controls and found that the study group healed an average of 11.7 weeks sooner. The 16 weeks interval chosen was a reflection of practice at the time with delays in

definitive closure with grafting recommended at least two weeks after this. Current standards demand closure within the first 72 hours[109] and, in addition, fixation techniques have become much more robust. In their series, Blick[105] had most treated in an external fixator followed by a period in cast. However their attempt to rationalise the use of autologous bone graft to prevent unnecessary delay of treatment based on definition[13] remains but there is still no clear guidance as to the optimal time to intervene particularly with modern technologies and practices remain varied. There is little further in terms of comparing times to intervene and the principles established by Blick *et al* are still widely quoted. Marino[20] concentrated mainly on the technical aspects of bone grafting and this is likely to be because, with the paucity of publications, there is little to discuss regarding timing.

BMP2 has been trialled in fractures at high risk of non-union at the initial procedure. BMP2 with reported success initially [96]. There were confounding factors in this study and when these were controlled for no advantage was seen[110].

1.6.6 Alternative therapies in the treatment of fracture non-union

Alternative therapies that would obviate the need for any surgical intervention thereby avoiding all the described risks remain an attractive prospect. Attempts at this are not new; Physick [111] in 1803 treated a humeral non-union by means of a percutaneously passed seton (silk threaded needle). There are now several techniques licensed for use in clinical practice but although the industry for these alternatives is estimated as being worth over $1 billion[20], supporting evidence remains incomplete.

Low intensity pulsed ultrasound treatment represents the bulk of the market in non-operative strategies and in 2006 was worth £350 million in the US alone[112]. Busse *et al* published systematic review of randomised controlled trials on the use of low intensity ultrasound on fractures[112]. Only one trial reported specifically on non-union[113] and although they showed a positive effect it was underpowered. Having reviewed 13 studies, Busse and co-workers concluded that although use of low intensity ultrasound was common the evidence was only of low or moderate quality. The better trials found no significantly positive effects. Another review in one of the main orthopaedic journals concluded that the evidence was overwhelmingly positive [114] for the use of low intensity ultrasound but did not include negative studies. The reason for the technique's popularity and it widespread use is probably best summed up by Jingushi *et al* [115] when they concluded that it "has been shown to be effective without causing either serious invasiveness or any undue risk". Other modalities (electrical stimulation, hyperbaric oxygen) have also been tried with varying popularity.

Again the evidence is at best weak [116-119]and large, properly conducted trials are necessary to demonstrate efficacy.

Further on from these non-operative strategies are those that try to achieve healing, like Physick, by percutaneous means. These aim to avoid open surgery by injection of growth factor concentrates directly into the non-union site. Hernigou *et al* have established a very interesting body of work looking at the mesenchymal capacity of marrow in patients with fracture non-unions[120-121]. Around this they have developed a technique of aspiration and concentration of iliac crest marrow using relatively simple methods and injecting this into non-union sites with reasonable reported success[122-123]. This in theory places a concentrate of both growth factors and mesenchymal progenitor cells into the non-union. The technique has been reproduced in a few cases in other centres [personal communication Vrahas, M, Massachusetts General Hospital, Boston, US]. There are technical difficulties (appropriate concentration, harvest of the correct cell lines and injection into a very small, closed space) and larger series are required. A similar technique, platelet rich plasma injection, has been commercially marketed for many musculoskeletal indications including fracture non-union. Again evidence remains preliminary[124] .

1.7 Conclusion

Fracture non-union remains an unsolved, significant clinical problem. Consensus on definitions remains elusive and the longstanding classification appears to be misquoted in most of the contemporary literature. There remain many unanswered questions with regard to the nature of the process itself and the tissues involved but because of the heterogeneity of the patient population and fracture site characteristics clinical studies are difficult. The current treatments are effective but are associated with considerable morbidity and are expensive. Many interesting and innovative techniques are emerging but the levels of evidence supporting them remain inadequate and most appear to add a level of expense. These studies though continue to evolve the understanding of the processes of fracture healing and the characteristics of the non-unions.

Chapter 2: Characterising the gap tissue in human fracture non-union

2.1 Introduction

There are difficulties in definition and classification of fracture non-union. The most utility definition is that of Frolke[14] that defined non-union as a point in the fracture healing process where focused and accurate treatment was required in order to achieve consolidation. Classifying the non-unions is more difficult. Much of what is currently described as 'atrophic' non-union[20] was classified as 'oligotrophic' by Weber and Cech in their original monograph[16]. Their scintigraphy studies showed activity that they interpreted as biological potential. It is similar to the vascular and metabolically activity demonstrated in the work of Brownlow and Reed [125-127] in an animal model of atrophic non-union. This latter group then went on to show that vessel density was similar in samples from human hypertrophic and atrophic non-union tissue and healing fractures. It is not clear that the atrophic tissue was strictly classified according to the original Weber and Cech work. Mesenchymal cells demonstrating pluripotentiality have been isolated from 'hypertrophic' non-union tissue[128] further confirming the assumption of biological potential by Weber and Cech and better defining that potential. Hofmann *et al* [128] published a very robust cellular and RNA analysis on cells extracted from the endosteal cancellous bone adjacent to 10 human hypertrophic non-unions. They compared these to similar samples from patients undergoing implant removal after uneventful fracture healing and found that the cells from the hypertrophic non-unions had reduced viability, differentiation potential and gene expression in culture compared to those from normal subjects. The implication was that although the cells from the non-union could demonstrate osteoblastic potential *in vitro,* their biological capacity *in vivo* was limited. Seebach *et al* [129] followed up the earlier work of Hernigou *et al* [120-121] showing that the numbers of mesenchymal colony forming units (i.e., the osteoblastic potential) was greatly diminished in bone marrow aspirate from patients with established fracture non-unions. They were the first to suggest that non-union had systemic manifestations. These data suggest a biologically complex environment and underestimation of this may account for the disappointing translational results [75]. There remains the assumption that the gap tissue is at best inert or even inhibitory and recommendations are for excision[21]. Indeed, in the Hofmann work this tissue was carefully removed and discarded.

2.2 Aim

The work presented here sampled this non-union gap tissue in patients undergoing augmentation or revision of the fixation of fracture non-unions and examined the genetic

profile and cellularity. The primary aim was to determine if it was feasible to profile the gene activity of the non-union tissue pertinent to fracture healing.

2.3 Hypothesis

The hypothesis was that there would be sufficient cells in sampled non-union tissue to profile activity in the genes related to fracture healing.

2.4 Materials and Methods

Inclusion Criteria:

Samples were obtained from 5 consecutive patients presenting to Vancouver General Hospital with a fracture non-union. This number was chosen to accommodate the time constraints of this feasibility study. The definition of non-union used was that suggested by Frolke *et al* [14]: "a disturbance of normal fracture healing with the expectation that no consolidation will be achieved without focused and accurate treatment". In addition, the diagnosis was not made until 6 months had elapsed from the index or previous intervention. Exclusion criteria were: infection, tissue from known HIV, Hepatitis B or C patients, and generalized inflammatory conditions.

Samples were taken as part of the standard surgical approach and no additional approaches were carried out to obtain samples. Local ethical approval was obtained (ethic number: H07-01921) for the study and all participants gave informed consent separate to that for their procedure. Three or four samples were taken from each patient. These varied in size according to the site and the anatomy of the non-union. They ranged between 27-64 mm^3 approximately. One or two samples were placed in formalin, fixed in paraffin embedded in wax and sectioned. The other samples were macerated under sterile conditions using a scalpel, placed in RNA later then stored at $-70^{o}C$ for mRNA analysis.

mRNA analysis

The samples were thawed to room temperature, further macerated in the RNAlater solution using two scalpel blades then placed in an eppendorf tube. This was centrifuged at 8000 x G for 10seconds and the excess RNAlater solution removed with a pipette. 1ml of TRIzol reagent (Applied Biosystems) was added to lyse the sample and vortexed until emulsified. 0.2ml of chloroform per millelitre of TRIzol was then added. The cap was secured and the solution shaken vigorously for 15 seconds and transferred to a water bath where it was incubated at $25^{o}C$ for 3 minutes. Following this the tube was centrifuged at 12000 x G for 15 minutes, separating the homogenates into the lower red phenol/chloroform base, an interface and an aqueous upper phase containing the RNA. The upper aqueous phase was removed

with a pipette taking care not to disturb or inadvertently draw up any of the interphase. The RNA was then cleaned and retrieved using the RNeasy spin columns (Qiagen). The final elution was placed into 30µl RNAase free water. The RNA was quantified using the microdot photospectrometry system. cDNA cloning was carried out using the high Capacity cDNA Archive kit (Applied Biosystems) using 20µl of 80ng/µl mRNA for each sample. The standard mix was prepared using 7µl of each sample and again quantified using the microdot system. The cDNA was then archived at -20 °C.

Probes were obtained from Applied biosciences for the genes listed in figure 2 and the qPCR carried out using the Taqman Fast Universal kit (Applied Biosystems).

Table 3 Target genes to assess biological activity of non-union tissue

All probes purchased BD Biosciences (http://www.bdbiosciences.com/services/customtechnologyteam/).

Osteoprotegerin (OPG), Rank Ligang (RankL), Bone Sialoprotein (BSP), Osteocalcin (OCN), Osteopontin (OPN), Collagen Type I (COL1), Collagen Type II (COL2), Collagen Type III (COL3), Collagen Type X (COL10), TNF receptor associated factor 6 (TRAF 6), Tartrate resistant acid phosphatase (TRAP), Cathepsin K (Cath K), Transcription factor SOX 9 (SOX9), Osterix (OSX), Alkaline Phosphatase (AlkPhos), Adipocyte protein 2 (AP2), Peroxisome Proliferator-activated Receptor 2 (PPAR2), Peroxisome Proliferator-activated Receptor Gamma (PGAR).

Marker for	Gene	Product no.	Comments
Osteoclastic activity	OPG	HS00171068_ML	Control osteoclastic activity. Produced by osteoblasts
	RankL	HS00243519_ML	
Non-collagenous proteins	BSP	HS00173720_ML	Proteins associated with ordered hydroxyapatite deposition
	OCN	HS00609452_GL	
	OPN	HS00959010_ML	
Collagen	COLIα1	HS00164004_ML	Type III collagen associated with repair tissue. Type X collagen associated with hypertrophic chondrocytes
	COLII α1	HS00264051_ML	
	COLIII	HS00164103_ML	

	α1		
	COLX α1	HS00166657_ML	
Osteoclasts	TRAF6	HS00371508_ML	
	Cathepsin K	HS01080388_ML	
	TRAP	HS00356261_ML	
Chondroblasts	SOX9	HS00165814-ML	Combined with collagen II as marker of chondroblastic activity
Osteoblasts	OSX	HS00541729_ML	Combined with collagen I as markers of osteoblastic activity
	Alk Phos	H500758162_ML	
Adipoblasts	PPARγ2	H01115513_ML	Markersof adipoblastic activity
	AP2	H00609791_ML	
	PGAR	H01101127_ML	

Histology

The paraffin embedded specimens were sectioned at 5 μm intervals and stained using H&E, von Kossa, TRAP and Masson Trichrome. Samples were processed courtesy of Margaret Luk, Pathology Department Vancouver General Hospital.

2.5 Results

Samples of non-union tissue were obtained in five patients. There were differences found in the consistency of the tissue. Some samples were grainy and difficult to macerate with a scalpel while other samples were soft and fibrous and easily macerated. Two samples from the one patient had different handling properties. Half of each sample was used for histological analysis and half for RNA extraction.

Table 4 Sites of non-unions from which samples taken

Sample	**Site**
1	Tibia
2	Femur
3	Clavicle
4	Femur
5	Femur

2.5.1 RNA analysis

Only very small amounts of nucleotide were isolated from the samples. This had been anticipated in the study design and therefore only a limited number of genes were examined. The cellularity of each sample was controlled for using the18s rRNA housekeeping gene and showed great between samples (Figure 1). The 18s rRNA figure shows the total quantity isolated in nanograms. This gene has been used in other studies looking at mesenchymal tissue and shown to be robust[130-131]. The variations therefore reflect differences in cellularity between specimens. In addition, two samples from one patient (ANU01 and ANU02) underwent separated RNA extraction and also showed variation. Figures 2-7 below represent the mRNA shown as a ratio of the quantity of mRNA obtained relative to the cellularity of the specimen (i.e. normalized to the 18s rRNA housekeeping gene so that it is more representative of the gene activity).

Figure 1 18s quantities in the non-union samples

Y axis represents the quantity (ng) of 18s RNA extracted from each sample. This was used to provide an estimate of the cellularity of the specimen. This showed considerable variation across the samples.

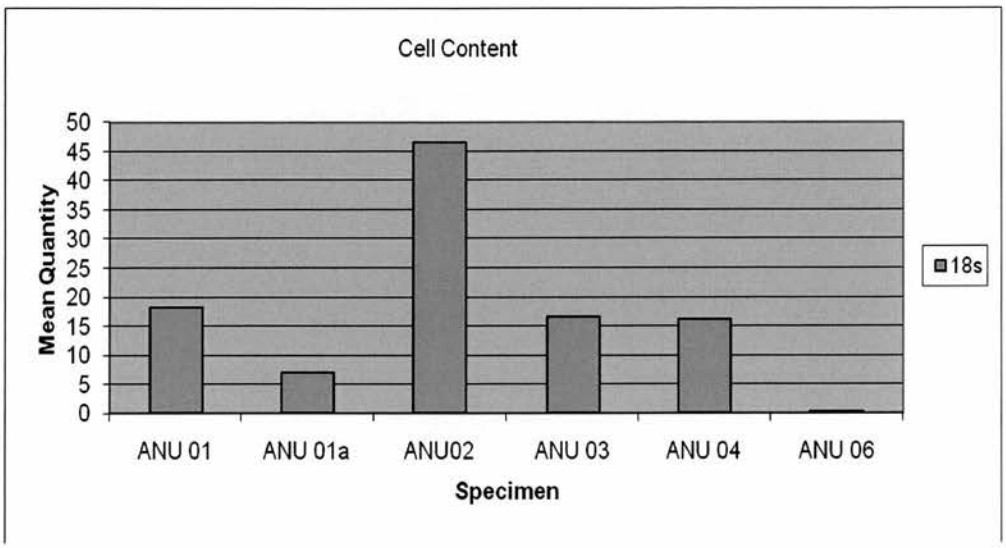


Figure 2 mRNA expression in all genes examined

Represented as a ratio of gene activity relative to 18s activity to control for differences in cellularity between specimens. Therefore the higher the value the more active the gene.

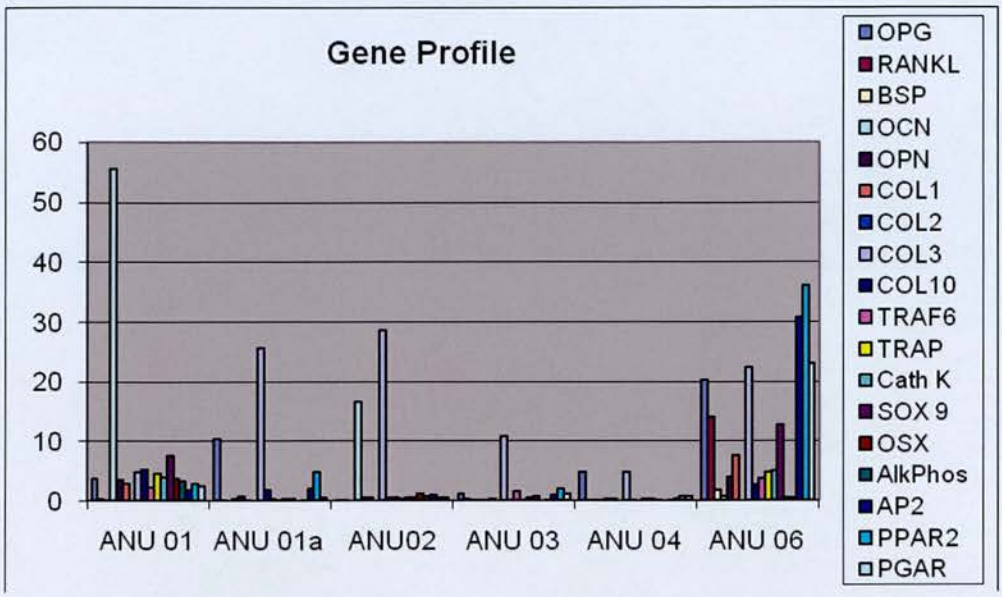


Figure 3 Osteoblastic activity

The genes that have been reported as to be relevant to bone formation are: were bone sialoprotein, collagenI, osterix and Alkaline phosphatase.

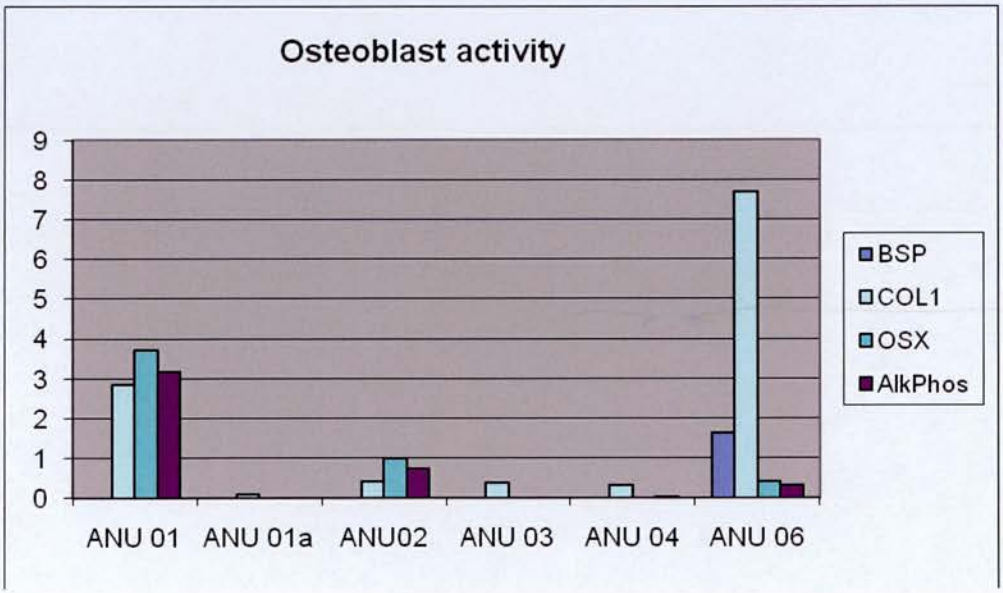


Figure 4 Osteoclastic activity

The genes examined were osteoprotegerin, rank ligand, TNF receptor associated factor 6, Tartrate resistant alkaline phosphatase and cathepsinK.

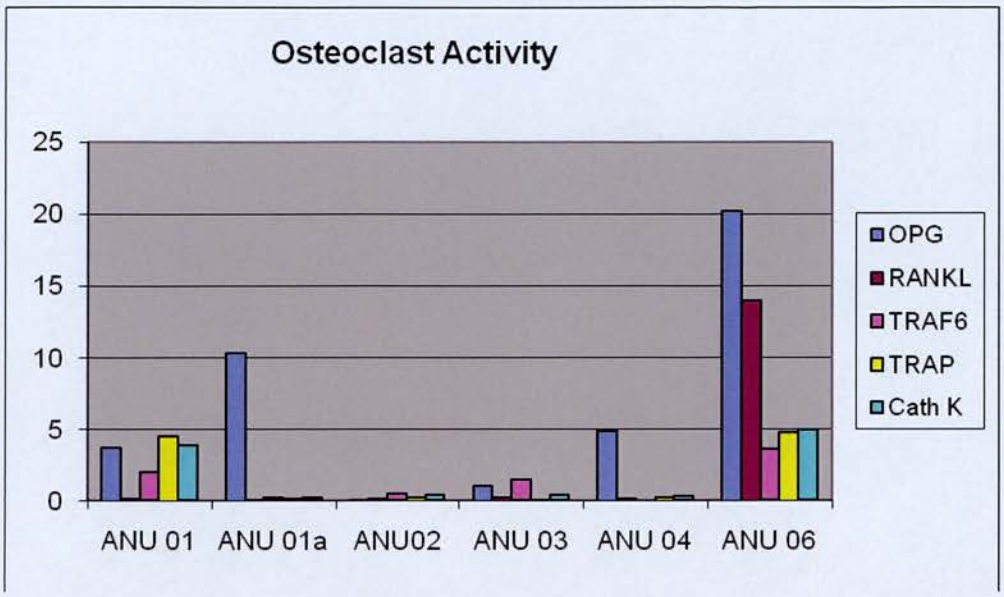


Figure 5 Collagen profile

Collagen I is associated with bone, Collagen III with repair or immature tissue while collagens II and X are associated with cartilage.

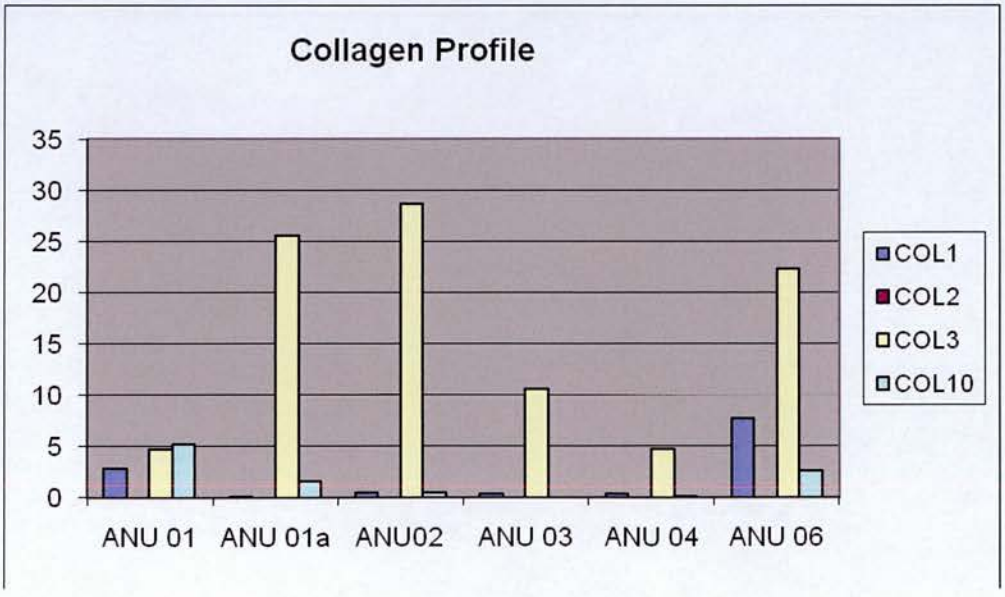


Figure 6 Chondroblastic activity

SOX 9 is closely associated with chondrogenic activity. Collagen II is also indicative of this.

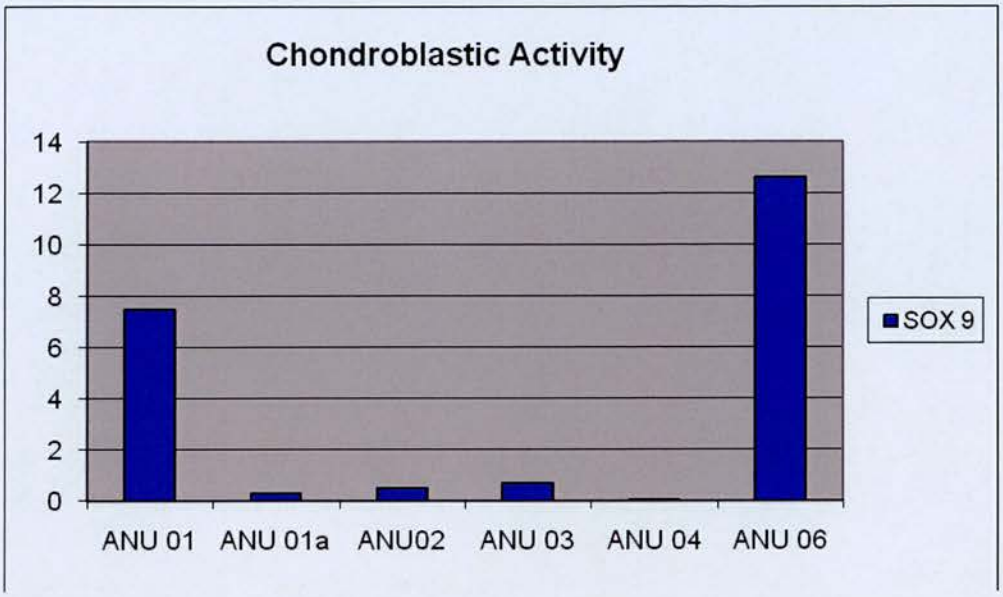
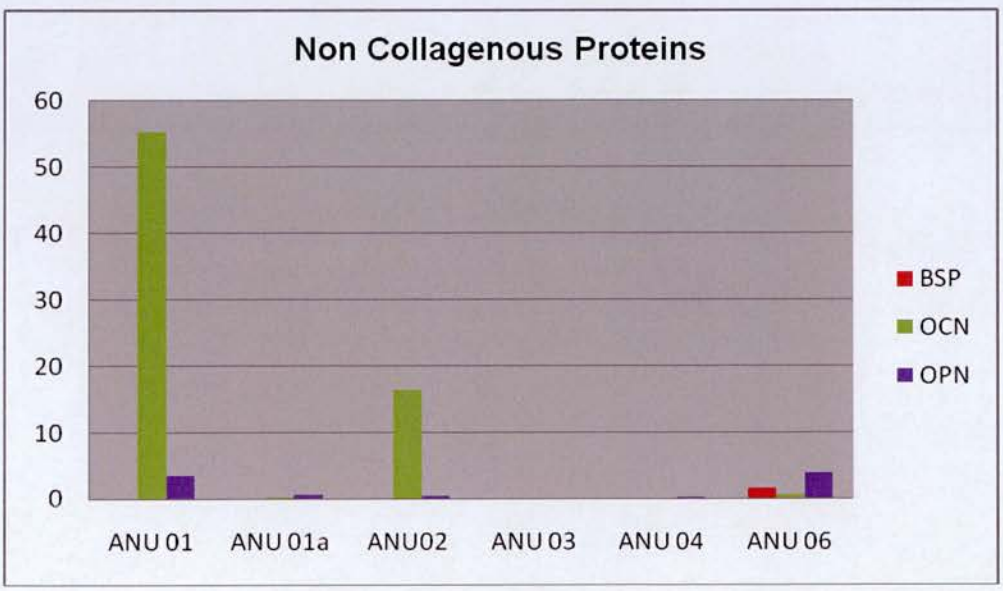


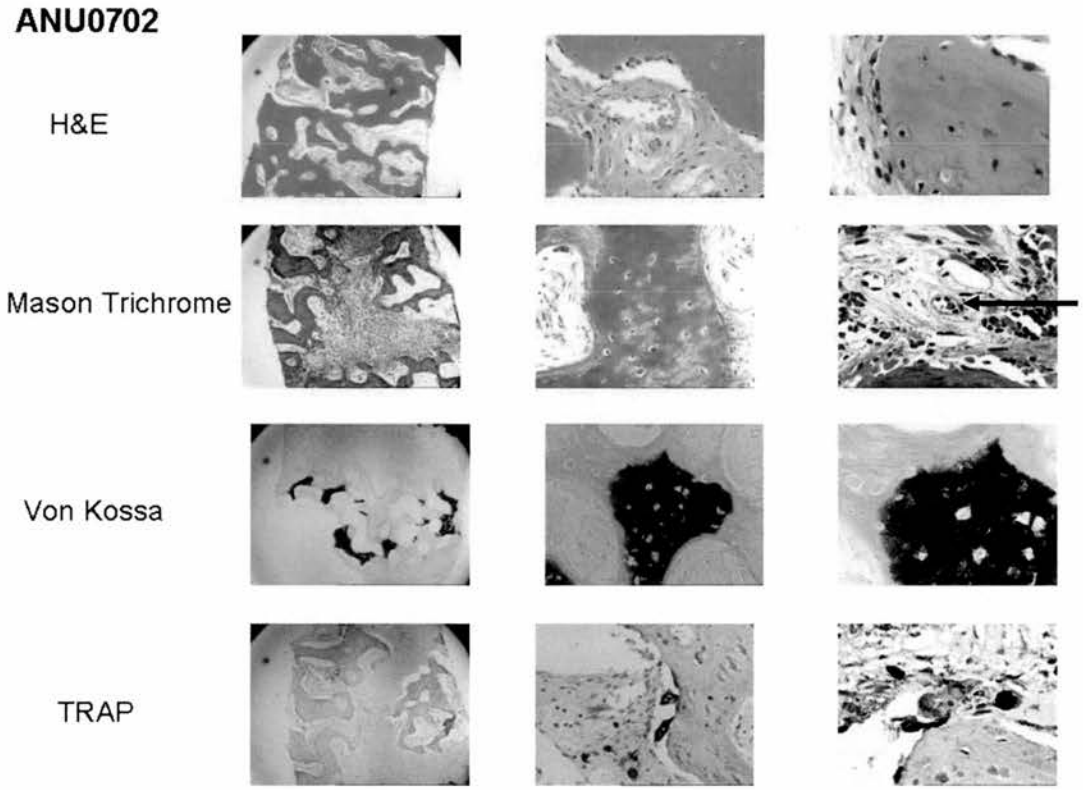
Figure 7 Non-collagenous proteins

These proteins are associated with a normal mineralisation process.(Bone sialoprotein, osteocalcin and osteopontin)



2.5.2 Histology

Images below are from left to right x25, x200 and x400 magnification respectively. They are illustrative of the architecture of the non-union tissue. The H&E stain showed areas of bland, unmineralised and cell sparse matrix separating cellular islets of woven bone. The Masson-Trichome demonstrated the relations of the fibrin/cellular areas (red) to the collagen/cartilaginous scaffold (blue). Nuclei stain blue/black. It also showed blood vessels within the cellular areas arrow). The von Kossa stain shows areas of osteoblastic activity (brown) which appears to correspond to the cellular areas within the relatively acellular matrix. The TRAP stain showed areas of osteoclastic activity (brown) and proved difficult for technical with loss of adherence of the sample to the slide in the initial sections. It did show activity limited to the edges of the cellular areas that had the appearances of woven bone on the H&E stain.



2.5.3 Clinical Progress

The images below are illustrative of the clinical picture as the non-unions progressed from their initial treatment.

X-ray 1 shows one view of a non-union of distal tibia with failure (breakage) of the implant. It is an apex anterior deformity with rounding off of the bone end suggestive of an 'atrophic' non-union. Other areas of the site show bone activity more in keeping with a 'hypertrophic' non-union. It was agreed clinically among three senior orthopaedic surgeons that this would not heal without intervention. X-ray 2 showed the healed non-union following posterolateral plating as a staged procedure to rule out infection.

X-ray 1

X-ray 2

X-ray 3 (below) shows a femoral non-union with an intramedullary nail in situ. The patients activities were greatly limited by ongoing thigh pain and he continued to used a walking aid. The medial side (left on the x-ray) has the appearance of an 'atrophic' non-union (Marino[20]) or an 'oligotrophic' non-union (Weber and Cech[16]). X-ray 4 (below) showed the healed non-union following plating. The patient was fully ambulatory without pain in his thigh.

X-ray 3

X-ray 4

2.6 Discussion

This study demonstrated that the there is a measurable gene activity in the gap tissue of fracture non-unions. The gap tissue was not excised in these patients and by the time the patient was considered healed clinically, it had ossified. Therefore while other published data has suggested a potential that is at best limited[120-121, 129, 132], it appears that the non-union tissue does retain adaptive capacity and can form bone. This is the clinical experience in the unit where this work was undertaken and underpins their approach to the management of fracture non-union. This work is pilot data but it supports the feasibility of relating the mesenchymal and osteoclastic activity of the non-union tissue to the histomorhometric architecture and to the clinical behaviour or the adaptive/healing potential of the fracture. Until now, osteoclast activity in relation to fracture healing and non-union has been largely ignored[133]. Much focus has been placed on translational research. This work has examined a clinical practice that seemed at odds with the published data[120-121, 129, 132] and examined it at the cellular level. This novel perspective highlighted a number of points.

Firstly, fracture non-union remains poorly defined[9] making understanding the pathology and rationalizing potential treatments even more difficult. The US Food and Drug Administration's definition of nine months from index incident is difficult to justify clinically when continuing pain and failure of return to function pressure earlier intervention. Therefore the pragmatic definition of Frolke[14] was used but it relies on clinical acumen and is therefore more difficult to standardise for the purposes of research. Classification remains a problem, most surgeons still divide non-unions into hypertrophic and atrophic varieties considering

them to be hypervascular and avascular types respectively[49]. Further, there is discrepancy in the literature between the original work of Weber and Cech[16] and that which is now classified as an 'atrophic' non-union[20]. Recent work that has shown 'atrophic' non-unions (avascular) to be as vascular as those with hypertrophic morphology (hypervascular)[125, 127] . However, it did not define which definition was used. This makes interpretation of their results more difficult.

Many non unions will therefore demonstrate evidence of both morphologies (e.g. ANU0701). The Frolke definition[14] circumvents the need for this tier of classification in that it establishes simply that there is a failure of consolidation that requires intervention. Gerstenfeld *et al*[134] suggested that the first step in approaching questions on fracture healing was a quantitative definition of the spatial geometry of the skeletal tissue morphogenesis during normal healing. In their animal model they demonstrated that this seemed to occur along the embryonic growth patterns. This study has not been repeated in human non-union tissue and was beyond the scope of this study. It might however explain the mixed morphological patterns in bones like the femora shown in this work.

Clinical studies have been hampered by the heterogeneity of the patient population, their injury patterns, treatments and clinical courses. This has been a major factor in attempts to produce reliable animal models where many of these events can be better controlled. These however can present a different set of problems[135]. Previous studies have not addressed the heterogeneity of the non-union tissue itself. Published work has focused mainly on the consistencies within the material and failed to comment on the heterogeneity. Wen *et al* [136] commented on the abnormal crystal and colloid structure that was present in all of their samples. Reed *et al* [127] also commented only on the similarities. In the work presented here, the tissue showed diversity in terms of the handling properties and gene profiles. These characteristics should be documented in future work. Cellularity, as estimated using the 18s rRNA gene, and gene activities also varied between samples. One similarity was that the mRNA extraction yielded only very small amounts of nucleotide in all the samples. Thus there are potentially extrinsic (between non-unions) and intrinsic (within the same non-union gap tissue) heterogeneities that need to be included in any analysis particularly when designing translational work.

The histological analysis showed areas that were largely acellular interspersed with cellular areas where the cell type and appearances suggested woven bone and blood vessels. An accurate histomorphometric mapping was beyond the remit of this study but the data here

suggests that this is feasible even with the small samples obtained. The size of the samples for this work was between 3-4mm in diameter and yielded the sufficient nucleotide for analysis and tissue for histology. The assumption is that the samples being contiguous are likely to share similar characteristics in terms of cell content.

The probes were selected to give an indication of mesenchymal (osteoblastic, chondroblastic and adipoblastic) and osteoclast activity and for the non-collagenous proteins associated with hydroxyapatite crystal deposition. It was felt that these probes were sufficient to provide an accurate, practical picture of cellular activity in the non-union tissue. Techniques, such as microarray may be useful in future work but the amount of nucleotide available from the samples may be the limiting factor.

Lawton *at al*[137-138] and Hofmann *et al* [132]have examined the osteoblasts at the bone ends associated with non-unions. The former group described an altered phenotype [137-138] and the latter correlated this to reduced proliferative and differentiation potential and decreased capacity for mineralization. The conclusions were that this was a pathological phenotype. It was not considered that it may be an adaptive and therefore physiological response. It should also be noted that in both groups the cells within the non-union tissue itself were not examined. The assumption is that the tissue has no inherent healing potential and in the Hofmann group[132] the gap tissue was carefully removed. This bias contrasts with observations in this study where the non-union tissue was left and had ossified by the time it was judged to have healed clinically. This suggests that despite the reported degenerative nature of the osteoblasts [132] and the paucity of colony forming units [121, 129] in the fracture non-union patient, the tissue remains adaptive enough to heal. It is not possible to tell from the current data whether the cells within the non-union tissue or cells recruited systemically and/or locally were responsible for the healing.

Collagen III was expressed in all samples very much in keeping with the accommodative nature of the tissue and with the work of Lawton *et al* [138]. Interestingly, collagen X was expressed in two of the samples and appeared to correlate with Sox9 expression, a chondrocyte marker. Collagen X is a marker of hypertrophic chondrocytes [139-140] and may suggest senescence of the cartilaginous tissue in the non-union site. There was no evidence of collagen II expression. Collagen I expression also appeared to be low across most of the samples suggesting a steady state. Collagen type 1 is known to have a low turnover in normal bone[139] which could account for the low expression in the samples.

This work has demonstrated the feasibility of quantifying the metabolic activity in small samples of non-union tissue and the potential to relate this to histological appearance and clinical behaviour. It showed gene activity suggestive of osteoblastic and osteoclastic metabolism in the non-union gap tissue and that there was observed heterogeneity between patients and within different areas of the non-union site. Correlating gene expression to histology and clinical activity would have major benefits both clinically and with regard to research.

Chapter 3: Model of fracture 'atrophic' non-union

3.1 Introduction

Roach *et al* in their excellent critique of experimental approaches [135] advocate that the level of question determines the model. For fracture non-union the level is that of the organism because the individual constituents appear to behave differently in isolation that when a composite in vivo. The fracture non-union patient population is very heterogeneous and this is one of the major problems with assessing clinical interventions. Animal models allow standardisation of the host and fracture non-union characteristics but present other difficulties [141]. Small animal models are cheaper and quicker but they have greater healing potential and osseous union is more difficult to prevent [135, 142]. Higher animals are closer to the human situation but are expensive and very time consuming to use. Even in large animals the physiological response to injury can be different to that of humans [143] creating problems when translating conclusions.

A rat model of an 'atrophic' fracture non -union was used for this study. This is a validated model that is analogous to the Weber and Cech definition of an 'atrophic' non-union. In addition, the interventions were carried out on established non-unions rather than in the acute phase. Again, this is analogous to the clinical scenario and it differs from the majority of intervention studies published[141, 144-148]. The non-union was created by the introduction of a gap that was less than half the bone diameter (i.e. non-critical) and stripping of the endosteum and periosteum to the same distance. No foreign material was introduced. The importance of this is that it recreates a comparable environment to the injury resulting from high energy trauma where the risks of non-union are greatest.

As has been stated, there are problems with drawing conclusions for clinical interventions from animal studies, particularly small animals. The latter tend to overestimate the efficacy of interventions. Therefore if an intervention is successful in a small animal model it may have some benefit in the clinical situation and there is a rationale for further research. If however, it fails to impact in the small animal model and the study design is robust then that particular strategy is unlikely to have an impact clinically.

3.2 Method

The model used for this project was of an 'atrophic' fracture non-union in a rat described by Reed *et al* [149]

3.2.1 Construction External Fixator

Materials

Nylon M16 washers

Nylon M4 nuts

Brass M4 nuts

Brass 30mm M4 screws

316LVM stainless steel, 0.5 X 35mm, cold drawn wire, bright anneal

Araldite instant clear glue syringe (Homebase, Product no. 234035)

Construction of ring mounting

A carbonfibre block was milled to provide a pedestal to mount four M16 nylon rings for accurate drilling (courtesy of John Lissimore, Biomedical Engineering, University of Edinburgh). Each fixator was therefore constructed as a set. A suspension of lubrication oil and water was used to cool the rings during drilling so that there was no thermal distorsion. The mould was mounted in the Turret CNC (computer numerical control) Milling machine and centred. A 4.2mm drillbit was used.

Drilling of holes

The holes were drilled in the nylon ring such that they were off centre. This meant that on removing a section the gap left could be varied to either wide or narrow. Two rings were cut leaving a wide gap and used proximally to leave room for the proximal calf musculature. Those with the narrow gap were used distally. The holes were drilled using distance from the centre of the ring calculated along two orthogonal planes. Therefore, the first hole was 11.55mm to the left. The bit then returned to the centre and was moved 10.47mm to right followed by 4.88mm perpendicular in a distal direction. Once the holes were drilled the unwanted sections were cut and the all edges chamfered.

Pin preparation

0.5mm pins were made to order by Alloy Wire International using 316LVM (medical grade) stainless steel. These were therefore of the same construction as K-wire and the fine wires used in Ilizarov and Taylor-Spatial frames. The wires were blunt on arrival. The point was created to approximate the 'Medin' design proposed by Piska *et* al.[150] using a fine grinding stone in a mounted minidrill. The pins were then cleaned and sterilised prior to use.

3.2.2 Surgical Equipment

Non-Sterile

Heating pad

2 X 7mm spanners

Philips screwdriver

Minidrill

Heavy forceps

Glue

Applicator stick

Vetwrap

Sterile

2X artery clips

Fine toothed forceps

Fine needle holder

Size 3 scalpel handle

Size 11 scalpel blade

0.8mm burr (round head)

2X pins bent as retractors

4/0 vicryl rapide

Vetbond

Saline

Preparatory solution

Betadine

Drugs

Hypnovel®

Hypnorm®

Vetergesic

3.2.3 Surgical procedure

Anaesthetic and Preparation of Operative site

A single intraperitoneal injection of a mixture of Hypnorm® and Hypnovel®, each at 1.15µl per gram body weight. The leg was then shaved using clippers and cleansed with Betadine solution.

Insertion of Wires

The bony anatomy of the leg and ankle was palpated. In particular the level of the ankle was noted. A variable speed foot control drill was used for all pin insertion. The point of entry for

the first pin was on the lateral border, just superior to the ankle .It was passed at a 20° angle to the tibia in an anterior-posterior lateral-medial direction. The next wire was passed 3mm above this but in a posterior-anterior direction, again at approximately 20 ° to the axis of the tibia. The third distal wire was passed anterior-posterior 4mm above the second. The fourth was passed posterior-anterior 3mm above the third. The tibia was templated against a dummy frame and the position of the top wire of the proximal set marked. These were then inserted as for the distal set but in a proximal to distal order. The external fixator was then attached to the eight wires as shown in the diagram. Each fixator was constructed to match the wire placement with care taken to avoid creation of tensile forces as these were attached to the rings. The wire ends were then covered in rapidly setting resin to secure their position.

Osteotomy

Once the fixator was secured the area of leg between the proximal and distal rings was again prepared using Betadine. A longitudinal incision was made and extended through the fascia. A retractor was inserted medially then laterally exposing 6-8mm of tibia. The scalpel blade was used to excise the periosteum for 2mm on either side of the osteotomy site. Care was taken to remove the periosteum posteriorly where the sides of the bone meet at a ridge. A 1mm osteotomy was created using the 0.8mm round headed burr. The gap was verified with a 1mm feeler gauge. The ends of the bone were cooled with saline throughout. The endosteum was stripped on either side by curettage using 23G needles bent at 90° so that the cutting edge could be used effectively anteriorly with one and posteriorly with the other. The fibula was fractured using three-point bending and the gap rechecked. The wound was then closed with 4/0 Vicryl rapide and Vetbond.

Following the osteotomy the wires were glued to the position to which they had been secured on the frame with the rapid setting Araldite resin. The whole construct was then wrapped with Vetwrap, taking care not to cause constriction on the hamstrings and leaving the wound area exposed for inspection.

Post-Operative Care

Immediately post-operatively, all animals received 0.25ml Bupenorphine. If there were any signs of distress this was repeated at twelve-hour intervals. The animals were wrapped, kept warm and observed until they had recovered from the anaesthetic. An initial weight was also taken. Thereafter they were checked on a daily basis.

3.3 Discussion

The rat model used in this work has been published previously and been shown to reliably produce an 'atrophic' non union reliably [149]. In this study there was a 5% rate of union, all of which occurred in the initial 4 weeks. No animals went on to heal if there had been no signs of osseous activity on the 4 week radiograph. All had further check radiographs at 8 weeks prior to any intervention to confirm this.

All animals began weightbearing on the operated limb from the second day post operatively and continued to use the limbs in a quadripedal gait pattern and in bipedal stance when exploring their surrounds throughout the experimental interval, again suggesting that the construct was stable.

Rats would often try to nibble the nylon rings as part of their self hygiene. Initially, several nibbled through the entire rings and were excluded. The problem was solved by reapplying the Vetwrap® on the second and third days post operatively after which the animals no longer ate the frames.

During the creation of the non-union care was taken to prevent thermal necrosis of the bone ends and all were checked for 'paprika' like bleeding at the end of the procedure. All gaps were checked with a feeler gauge and likewise for the level to which the endosteum and periosteum were stripped.

The operative time involved in constructing the fixator and operating on the bone ends as described was 1.5 hours. Intra operatively in several animals the saphenous vein was avulsed by the most distal wire. This led to a minor ooze at the time of surgery and some post operative bruising but there were no untoward effects. The morphology of the proximal tibia in the rat is triangular and in some this was very narrow necessitating a slight alteration in wire direction in order to achieve adequate wire purchase.

In one animal the self hygiene led to wound breakdown. This was monitored closely and healed in 6 days but the animal was excluded as infection could have complicated the non-union.

The time point for the intervention was set as 8 weeks in this study design. However, as no animal, which had a 4 week radiograph that had failed to show any bone activity, went on to heal, it would have been appropriate to use 4 weeks as an intervention timepoint in future work saving on time and costs.

Chapter 4: The osteogenic potential of non-union gap tissue

4.1 Introduction

The non-ossified tissue at the site of a non-union has been considered to be inert at best or an obstruction to healing and removed in the course of treatment[132] . However, more recent data challenges this view. Wen *et al* [136]examined humeral non-unions using electron microscopy and x-ray diffraction and showed scattered islands of hydroxyapatite scattered throughout the non-union tissue. Brownlow [126] demonstrated metabolic activity within the non-union material and the same group also showed that once a steady state had been achieved 'atrophic' non-unions had a vascularity comparable to the healing fracture [125, 127, 149]. They also demonstrated a similar pattern of vascularity in their *in-vivo* model, the suggestion being that hteir model had similar characteristics to human non-union gap tissue. Boyan *et al* [151] extracted the cells from the gap tissue in a canine model and were able to demonstrate *in vitro* pluripotential mesenchymal cell lines. Other groups [128, 132, 152] have now also shown that mesenchymal cells with osteogenic potential can be isolated from human 'hypertrophic' non-union tissue.

4.2 Hypothesis

The hypothesis was that there were cells present within the gap tissue with osteoblastic potential.

This was pilot work to determine if mesenchymal cells with osteogenic potential could be isolated from a validated rat model of an 'atrophic' fracture non-union.

4. 3 Method

The model used was created as described in the previous chapter and validated by Reed *et al*[149].

4.3.1 Harvest of the Non-union tissue

At the 8 week time-point the animals were euthanized using increasing CO2 levels and death verified by cervical dislocation. The failure of osseous union was confirmed on fluoroscopy. Under aseptic conditions the soft tissue in the fracture gap was harvested. The leg including the fixator was prepared using aqueous betadine. The skin was incised using the previous incision. This exposed the non-union between the bone ends. Blunt dissection was used to free it from the muscles posteriorly and medially. Sharp dissection was then used to detach

the gap tissue from the bone ends. This was placed into Ependorph tubes of sterile Hanks solution.

4.3.2 Preparation of the Gap Tissue and Cell Culture

The preparation of the tissue for cell culture was carried out using an aseptic technique in a designated clean air hood. The tissue was washed three times in phosphate buffered saline (PBS) then placed in a drop of trypsin and morsellised using a sterile No.15 scapel blade until a fine consistency was achieved. This solution was pipetted into a T25 culture flask in 3mls of standard media (DMEM with 10% Fetal Calf Serum (FCS), 1% streptomycin/penicillin and 1%glutamate). This was left for 3 days before the media was removed. The adherent cells were washed 3 times with PBS then replaced in 3mls of fresh media. Subsequent media changes were every 2-3days until day 21-28 when the cells had achieved approximately 90% confluence.

Once the cells were in a confluent monolayer the media was removed and the cells washed 3 times with PBS. 1ml Trypsin was then applied and the cells incubated at 37^0C for 10 minutes until the majority of cells had detached. The cells were then re-suspended in standard media and split into two T25 flasks. The cells were then cultured for a further 21days before being collected and counted. The resultant cells were plated into 24 well plates at a density of 10000 cells per well in 0.5ml media. Once the cells were confluent on in the wells they were divided into four groups. The control group was cultured in the standard media. All groups were culture for 21 days with the media changed every 2-3 days. In addition, prior to each media change the chondrocyte pellets were centrifuged at 150G for 5 minutes.

At the 21 day timepoint the plates were fixed in 4% paraformaldehyde. The chondrocyte pellets were placed in a drop of 5% PVA and frozen to -80°C.

4.3.3 Osteogenesis

Cells were cultured in an osteogenic media. This comprised: complete standard media with the addition of 50µmol/L Ascorbic acid, 10mmol/L β-glycerophosphate and 0.1µmol/L Dexamethasone.

Osteoblastic activity was shown by labelling with Alizarin Red S for bone nodules. This was chosen for pragmatic reasons and further work should also stain for alkaline phosphatise.

4.3.4 Chondrogenesis

Further cells were taken for chondrocyte culture. $2x10^5$ cells were also aliquoted into centrifuge tubes and spun at 150G for 5 minutes. The resulting pellets were washed in PBS three times then placed in chondrogenic media. This comprised: Glutamate 2 mmol/L,

Dexamethasone 0.1 µmol/L, Ascorbic acid 50 µmol/L, Na-pyruvate1 mmol/L, Proline 40 µmol/L, TGF$\beta_3$10 ng/ml and ITS+ Premix (final concentrations: Bovine insulin, transferrin, selenous acid 6.25µg/mL, Linoleic acid 5.3µg/mL, Bovine serum albumin 1.25mg/mL). The chondrocyte pellets were sectioned on a cryostat and stained with Safranin Red O.

4.3.5 Adipogenesis

The remaining cells were cultured in an adipogenic media. This comprised: complete media with the addition of Dexamethasone µmol/L, IBMX 500 µmol/L, Insulin 1 µg/mL, Indomethacin 100 µM.

Oil Red O was used to stain for adipocytes.

4.4 Results

The gap tissue proved to be quite delicate and very small samples were obtained (1-3.3mm^3 approximately. Here samples were used to refine the harvesting and culture method as shown in table one. Five animals were used for the experiment as shown in table 2. Only 3 samples provided enough cells for final analysis (60%).

Table 5 Samples used to establish optimal extraction and plating technique as recorded in the methods section.

Number of samples	Outcome
n=1	Handling error leading to loss of cell in the extraction process (collagenase used)
n=2	Trypsin time thought to have been too long with poor adherence of cells to flask and cell loss at first media change

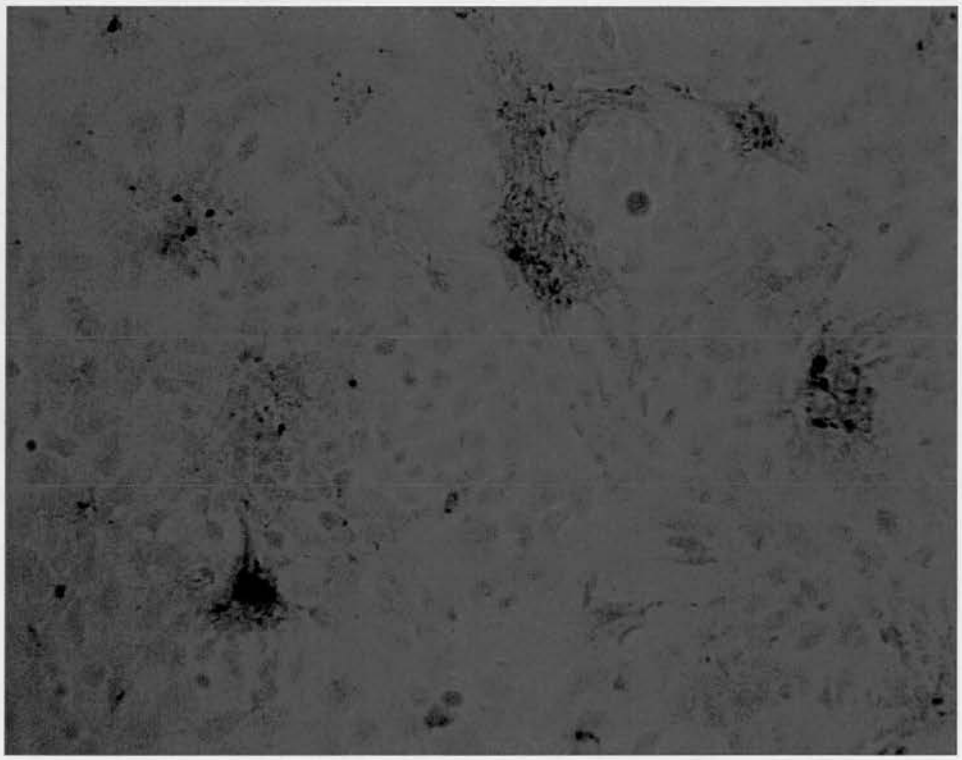
Table 6 Samples used in final cell culture

Number of samples	Outcome
n=1	90% confluence not achieve from initial culture
n=1	Failed to achieve confluence after first passage
n=3	Confluence achieved after first passage

	and sufficient cells for final analyses (10 000/well)

Osteogenic potential

The alizarin showed a degree of calcification in the control media but not in a nodular pattern. Staining of the adipogenic wells with alizarin was negative. In the osteogenic wells, the nodule pattern and the positive staining with alizarin were compatible with there being osteoblastic activity present.

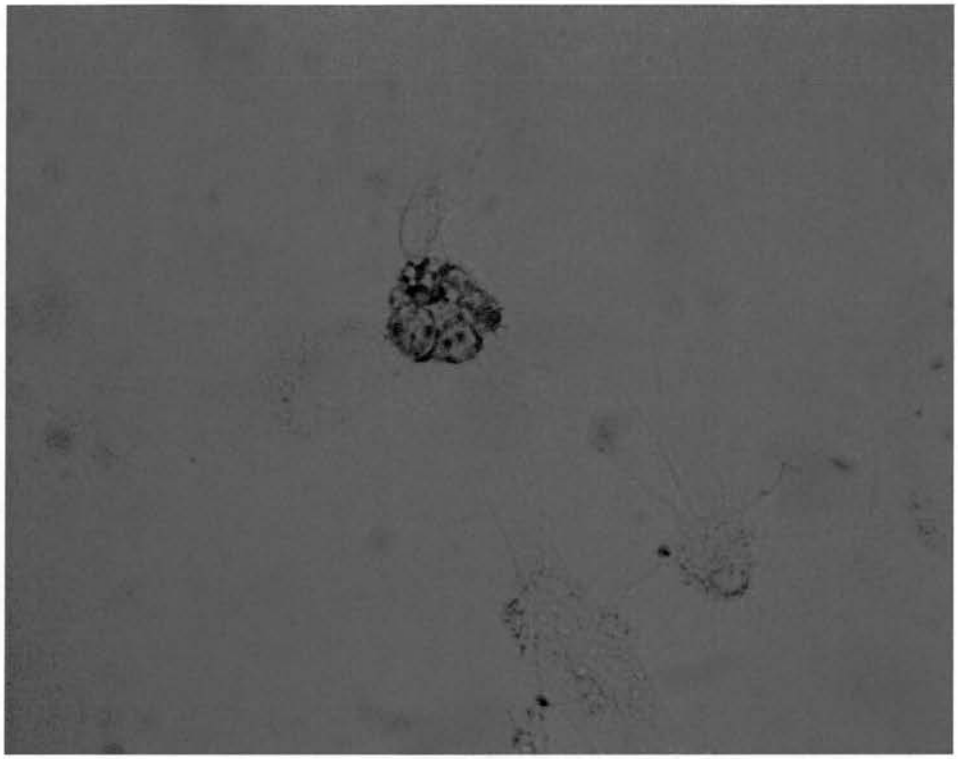


Chondrogenic potential

The pellets stained positively for Safranin Red O, supporting the presence of chondrogenic activity.

Adipogenic potential

Staining with Oil Red O In all the wells was negative.



4.5 Discussion

The results presented here support the assertion that gap tissue from the site of an atrophic fracture non-union is not inert. The vascularity, metabolic activity and cell turnover [126] of human atrophic non-union tissue and that of the gap tissue in the model used for this study has already been demonstrated [149]. The work presented here has shown that the tissue contains cells that retain the *in vitro* capacity for osteogenic activity. These findings provide a rationale basis for interventions that could potentially drive the intrinsic material in an osteoblastic direction to achieve union. The work also showed that the tissue was relatively delicate as was also observed by Boyan *et al*[151]. Once the procedure had been refined only four of the samples had sufficient cell division to allow a passage and only 60% had sufficient growth for the final analyses.

A lag in revascularisation of the non-union site relative to normal fracture healing [125, 127, 149] has been noted. The other markers of metabolic activity have also shown temporal lags [126 153]. It has been hypothesized that the factors not being present at the appropriate time contributes to the formation of the non-union[126] , however the lag in appearance of the vascularity and metabolic activity could equally be argued to be a result of the non-union rather than the cause of it. Seebach *et al* [129] quantified the colony-forming units (cfu's) of mesenchymal cells in multi-trauma patients, those with a single fracture and those with a fracture non-union. Interestingly they showed the highest numbers in multiply injured patients. These result from high energy trauma and have the highest rates of fractures going on to non-unions. They also demonstrated gender differences. These data suggest that the reasons for failure of osseous healing are more complex than lack of stem cell capacity or lack of growth factor stimulation. The work by Seebach *et al* suggests that in high energy trauma the systemic biological capacity for healing is higher than uninjured volunteers and therefore further stimulation in the acute phase may not lead to a further increase in activity and subsequent decrease in non union rate.

Osteogenic potential has been demonstrated in the gap tissue from a canine mode[151]. This model used a 3mm defect created in the midshaft of the radius with an oscillating saw. This reliably fails to heal and although a fibrous defect is created, the magnitude of the defect mean it is less clinically analogous as this gap size would not be accepted in clinical practice. The work presented here confirmed that a similar potential was present in the tissue from a validated, more clinically relevant model of an established atrophic non union. Work

published after this research undertaken has subsequently also demonstrated that cells retaining multipotential mesenchymal properties can be isolated from human hypertrophic non-union tissue [128, 152]. This would suggest that the same potential is present in human hypertrophic non-unions. It would be interesting to compare tissue from the non-union models to that of humans with regard to osteoblastic potential. This was beyond the scope of the current study.

The osteoblastic potential was explored by Lawton *et al* [137-138] who suggested that osteoblasts associated with a non-union showed atypical phenotypes. Hofmann *et al* [132] have examined this further and found that these osteoblasts also have reduced viability and replication potential. In addition to altered biology there is also evidence that the reservoir of osteoblastic cells is depleted in chronic fracture non unions [121, 123, 129].

Altering fracture mechanics by augmentation or substitution of the fixation method can lead to successful healing of fracture non union without exposing or intervening at the non-union site[16]. This suggests that with additional control of the forces across the tissue, the biological drive returns to ossification. It raises the question as to whether driving the biology alone could be sufficient to increase the intrinsic stability of the non-union site and provide conditions conducive to ossification.

This work suggests that there is tissue within this complex biological system that can demonstrate pluripotentiality and cells that can proliferate given the correct conditions. Clinical experience suggests that the tissue can remain adaptive in keeping with the experimental findings, provided that the appropriate mechanical environment is present.

Chapter 5: AdBMP2 and TP508 treatment of atrophic fracture non-union

5.1 Introduction

AdBMP2 (i.e. the BMP2 gene construct with an adenoviral insertion vector) has been shown to be effective in achieving osseous bridging of critically sized defects in a rat and rabbit model[99, 101, 104]. TP508 has shown similar potential in a mouse and rabbit models[76, 80, 89]. This study evaluated these orthobiological agents in a rat model of established 'atrophic' non-union. The latter model is more analogous to the currently used definition of an 'atrophic' non-union[20]. The principle outcome measures for this study were radiographic and mechanically confirmed osseous union. The secondary endpoints were histological analysis for vascularity and osteoblastic activity. The hypothesis was that biological potential in fracture non-union tissue can be stimulated leading to osseous union by closed percutaneous injection of induction factors.

5.2 Method

The rat model of atrophic fracture non union was created as already described. The 8 week radiographs were performed in the standardised fashion. With the animal still anaesthetised and the leg secure in the jig, the non union site was cleansed with Betadine antiseptic solution. All injections were carried out using a 0.3ml syringe and 26G needle. The needle was introduced through the skin obliquely from the lateral side with the bevel facing the proximal bone end. The tip was advanced until contact was made with the bone then swivelled through 180° so that the solution was injected into the osteotomy gap. The test suspension was injected directly into the fibrous tissue at the non union site. The area was then cleaned again and the animal wrapped and recovered in its cage.

5.2.1 Adenoviral construct and Amplification

Aliquots of replication-defective, E1- and E3-gene deleted adenoviral vector encoding for BMP2 were kindly supplied by Professor. CH Evans, Center for Molecular Orthopaedics, Harvard Medical School. This stock virus was amplified on HEK 293 cells. The adenoviral particles were extracted and purified using the BD Adeno-X^{TM} purification kit. The infectious titre of the purified viral solution was calculated using the BD Adeno-X^{TM} Rapid Titre Kit then stored at -80°C. On the day of injection the AdBMP-2 was thawed and re-suspended as aliquots of $1x10^7$ and $1x10^9$ in 10µl PBS. This was kept on ice pending injection. The doses used were inclusive of the range of doses in previous work[99, 101].

6.2.2 Preparation of thrombinpeptide solution/suspension

TP508 peptide was supplied by Orthologic. It was supplied in powder form for use as a solution and as a microsphere scaffold loaded form for use as a suspension. The latter provided a controlled release that has been shown to be more favourable when the peptide is used in bone healing [81]. The solution and the suspensions were prepared using the manufacturer's guidelines and the doses used were the same as have been published in previous work[77, 81]-. The solution /suspension was prepared under sterile conditions using sterile phosphate buffered saline (PBS) so that the experimental concentration was in 10μL.

5.2.3 Groups

Each group consisted of 5 animals.

Adenoviral groups

Group I	AdBMP2 at $1x10^5$
Group II	AdBMP2 at $1x\ 10^7$
Group III	AdBMP2 at $1x10^9$
Group IV	AdLacZ at $1x10^5$
Group V	AdLacZ at $1x\ 10^7$
Group VI	AdLacZ at $1x10^9$

Thrombinpeptide groups

Solution	Group I 1μg
	Group II 10μg
	Group III 33μg
Microspheres	Group I Low dose
	Group II High Dose
Control	No intervention

5.2.4 Radiographic assessment

Radiographs were taken at time point zero (8 weeks following the initial surgery to create the non-union), the time of the treatment injection. If there was any suggestion of osseous activity at the non-union site the animal was excluded from the trial. Further radiographs were then performed at the 2 week, 4 week and final 8 week time points to assess for bone formation. Radiographs were performed by fixing the frame in a specially designed jig. This was placed on the cassette containing the film at a fixed distance of 50cm from the source.

An exposure time of 0.5mS and a dose of 50mKVA were used. An anterior-posterior (AP) and oblique (O) (45°) were taken for each animal.

Radiographs were assessed using a visual analogue scale. All radiographs were graded independently by two experienced orthopaedic surgeons. The scale used is outlined in Table 7 below.

Figure 8 Samples of the Xrays at each of the time points showing that the non-union site failed to ossify.

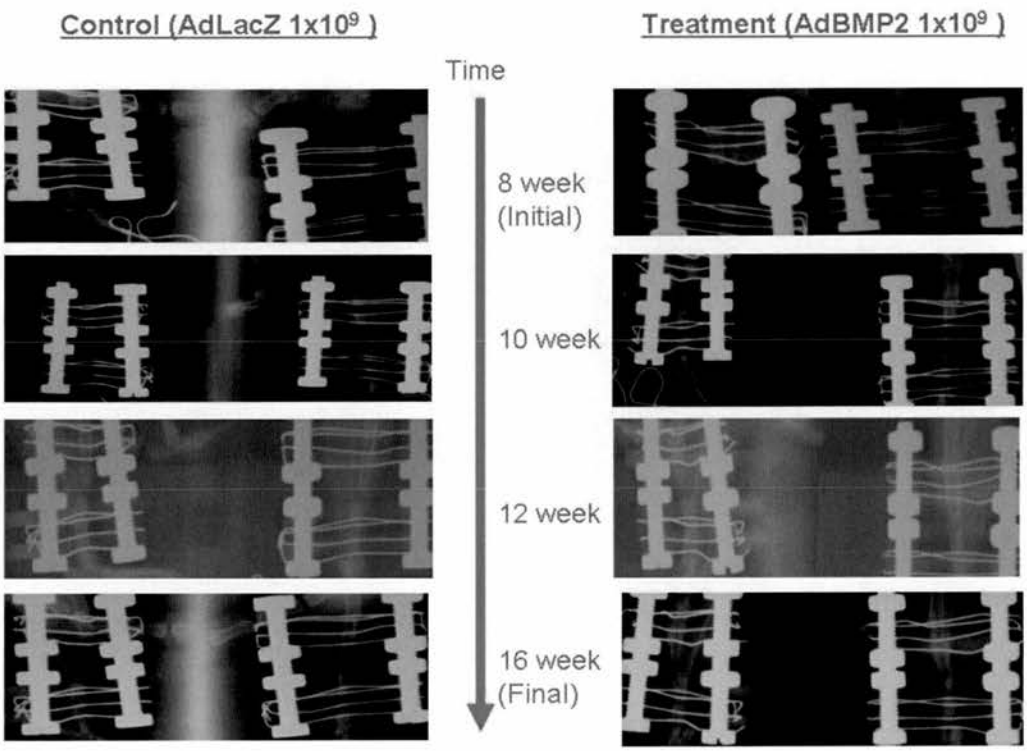


Table 7 Radiographic scoring system used to grade the radiographic evidence of osseous activity in the non-union site.

Radiographic findings	Score
No identifiable activity	0
Calcification in fracture gap	1
One cortex bridged	2
Two cortices bridged	3
Three cortices bridged	4
Four cortices bridged	5
Complete healing	6

5.2.5 Harvesting of non-union

At the eight week time point all animals were sacrificed using a chamber with a rising concentration of carbon dioxide in accordance with the Home Office guidelines. Final radiographs were obtained immediately. Using aseptic technique, in a sterile field, the skin overlying the non-union site was then excised. The tibia proximal and distal to the gap was exposed and osteotomised using the 0.8mm burr leaving a 2mm cuff of bone on each side of the gap. The construct was freed from the surrounding muscle talking care not to disturb the site of the non-union. It was soaked in 5% polyvinyl alcohol (PVA) for 5 minutes then placed in a freezing bath of hexane at -80°C for a further 5 minutes. The sample was then stored at -80°C until sectioning. 7μm sections were taken through the non-decalcified samples using a cryostat. They were captured on a glass slide and fixed in 4% paraformaldehyde (PFA) for 10 minutes followed by three five minute washes with PBS. The fixed sections were stored at 4°C.

5.2.6 Histology

Analysis was performed by Dr. Cristina Huber, Musculoskeletal Laboratory, Edinburgh using von Kossa staining to indicate osteoblastic activity and immunohistochemical staining using an antibody against von Willebrand factor to determine vascularity. The area of positive staining for von Kossa and the vessel count per high powered field across 5 fields were measured for each sample.

5.3 Results

All animals continued to display quadripedal gait and bipedal stance using the affected limb throughout the study period.

Xray

No difference was found between the control and treatment groups radiographically. All scored zero on the scale outlined above.

There were no differences between the different concentrations of AdBMP2. There was also no difference between the different concentrations and preparations of TP508.

Mechanical testing

Mechanical testing was performed in an earlier series to show that where there was no radiographic indication of healing (i.e a score of 0 on the scale above) there was less than 0.1N resistance to a bending force applied in a 3-point manner, i.e. there was no intrinsic mechanical stability and the sample deflected under its own weight.

Therefore, neither AdBMP2 or TP508 were effective in producing clinical/mechanical union or in creating radiographically apparent osseous activity.

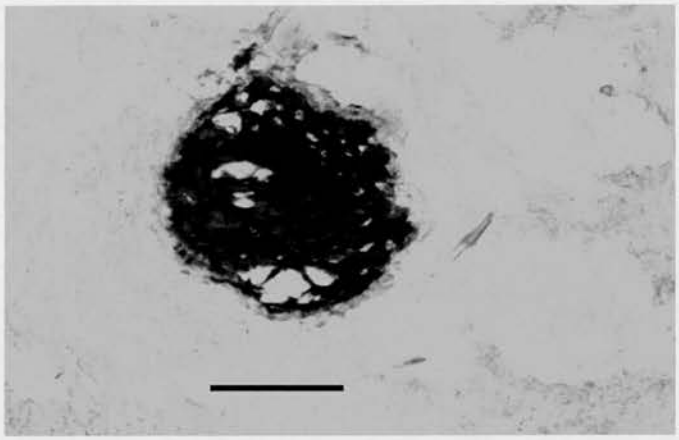
Histology

(Sections courtesy of Christine Huber)

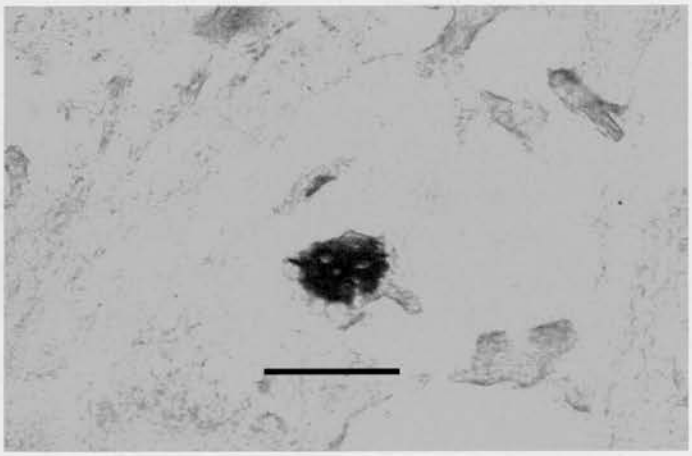
Figure 9 Von Kossa staining of the TP508 groups

This showed some evidence of bone nodule formation in the high and low dose TP508 groups (a and b) but not in controls(c). There was however no radiographic evidence of union in any group. Bar = 100μm x 200 magnification

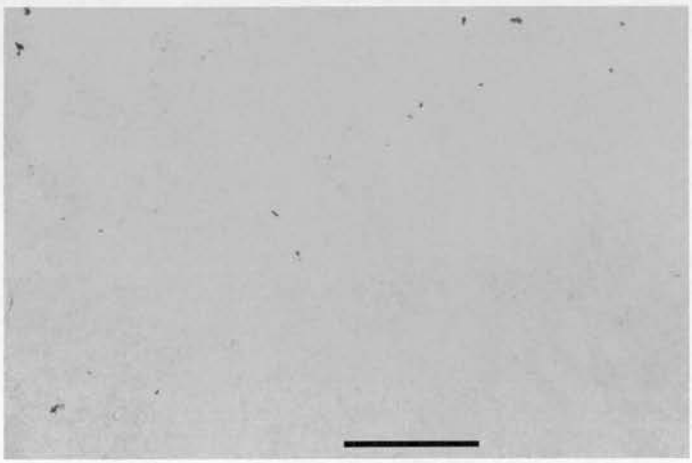
a . high dose



b. low dose



c. Control



In the TP508 high and low dose microsphere groups, there were increases of 43.9% and 9.9% in bone formation relative to the control group. There was also a statistically significant

difference in vessel density between the TP508 groups and controls but not between high and low dose groups.

5.4 Discussion

Both TP508 and AdBMP2 have been shown to be effective in stimulating ossification of critically-sized defects, fractures and the anlage produced by distraction osteogenesis in small animal models. The model in this study is a non-critically-sized gap model of 'atrophic' non-union and is more analogous to the current clinic definition[20]. The main outcome in this study was radiographic evidence of osseous activity or bridging bone. A radiographic assessment along with a clinical assessment (mechanical, functional, pain) is how union is determined in clinical practice. In contrast to the previous reports in rat[99, 101] and rabbit models[104], there was no evidence of clinically significant osseous activity in fracture gap in this clinically analogous model.

The methodology used the same preparation protocols and injection techniques that have been used in the other models [77, 81, 99, 101]. Betz *et al* [99, 101] demonstrated that the viral product was delivered into the non-union site and produced sustained quantities of BMP2 locally with negligible systemic effect. With regard to TP508, previous work has also shown that the preparation can be effectively delivered to the non union site by this method [77, 81].

The work presented here represents a further step from the gap models where both AdBMP2 and TP508 have been shown to be effective [76-77, 79-81, 89, 99, 101, 104, 154-155]. In the clinical situation, a non-union is often the result of both biological and mechanically unfavourable conditions. The model used here is representative of this situation.

In order to be useful in the treatment of fracture non-union, ideally the peptides needed to demonstrate efficacy in models of the established non-union situation. The test substrate was introduced into the gap tissue 8 weeks from the initial surgery. In almost all of the previous animal studies showing efficacy it was injected in very early in the post operative period. This may represent a more biologically favourable environment than the model used in this study. Clinically recombinant BMP2 has been used in this manner. However, the treatment is expensive and further evidence of its efficacy and cost effectiveness would be of value. [96].

The histological analysis of the TP508 microsphere samples demonstrated increased vascular and ossific activity. However, whilst these results are encouraging and were statistically significant, they did not represent a clinically significant improvement as indicated by an increase in union rate. At the time of this study, Orthologic were considering marketing

TP508 for the treatment of non-union. It had already been used in clinical trials in distal radial fractures [88]. Based partly on the preliminary results of this work, the marketing strategy has now been directed away from its use in the non-union situation.

The reasons for failure of healing in the current study are not clear from the available data. Although the techniques used were a replication of those from other successful studies, further work is required to demonstrate that the appropriate quantities of BMP2 and TP508 were delivered at the non-union site. Only then can the assumption be accepted that it is the biological environment that is the main contributory factor in the failure of union.

There are problems inherent in the long term treatment of fractures with external fixation devices in both patients and animal models. Wire loosening and breakage and infection leads to loss of mechanical integrity of the construct. The animals were monitored closely in the 8 weeks leading up to the intervention point. Radiographs were undertaken at 4 weeks. Overall, 5% showed signs of osseous activity and went on to heal by the 8 week mark. None of these animals were included in the study groups but this suggests that the construct was robust and maintained mechanical stability. No animals that had failed to show radiographic evidence of healing at 4 weeks went on to heal. In rats, loss of mechanical integrity of a limb leads the animal to exclude it in gait or weightbearing because of pain. All animals were checked on a daily basis by the investigator and also by the facility staff. All continued to exhibit quadripedal gait and bipedal standing. Where there were any concerns a home office veterinary surgeon was contacted and the animal euthanized. None of these animals were included in the final experimental groups. These data suggest that there was no major issue with mechanical stability of the construct.

Infection is one of the major causes of fracture non-union. If infected, there are signs of either suppuration around the pinsites or failure of the animal to gain weight. In addition to the animal checks outlined above, there were several groups used in a different experiments where the non union material was harvested at 8 weeks and used for cell culture. None of these cultures showed any evidence of infection. In the experimental groups therefore, no evidence could be found of infection contributing to failure of the interventions.

The model of fracture non-union used in this study is analogous in many respects to the clinical situation. However, it is a hostile environment in terms of osseous healing, probably much more so than many of the gap models employed to evaluate novel approaches to the treatment of non-union. This may indicate that percutaneous injection as a treatment of non-

union clinically may be more difficult than suggested by the work using small animal gap models. Further work is needed before this can be trialled as a clinical solution.

Chapter 6: Discussion

6.1 Evaluation the biological potential of fracture non-union gap tissue

This thesis has presented preliminary work aimed at characterising the biological potential and character of fracture non-union gap tissue. It began with an appraisal of the current literature on the classification and treatment of fracture non-union. It then detailed work that investigated activity in genes associated with osteoblastic and osteoclastic processes in human non-union tissue. Much that is understood about fractures and fracture non-union comes from animal models. In the subsequent chapters work was presented that used a rat model of fracture non-union to firstly, examine the osteoblastic potential of the gap tissue and evaluate percutaneously injected a viral vector (AdBMP2) and recombinant protein (TP508) as a method of driving the biology of the gap tissue to treat the non-union.

6.2 Definition

Defining when a fracture has healed remains problematic. Corrales *et al*[9] could not find a consensus on the definition of fracture healing when they reviewed the publications from the main orthopaedic journals. Bhandari *et al*[10] published similar findings when they surveyed the international orthopaedic community. This makes definition of fracture non-union even more problematic. The FDA definition[13] is widely quoted and provides a standard but that of Frolke[14] is pragmatic and aligned to clinical practice. It is the latter definition that a non-union was 'a disturbance of normal healing with the expectation that no consolidation will be achieved without focused and accurate treatment' that was used in this work.

6.3 Classification and treatment

The classification of fracture non-union still relies on the system proposed by Weber and Cech in 1976[16]. Others that have been published[17-19] but are adaptations or derivations of this system. Is a classification proposed over 35 years ago still relevant? Examination of their work would suggest that most of their proposals are supported by recent vascular studies[125, 127], cell biology[128, 151], growth factor and RNA analyses[126]. In addition, despite their cautions, the biological potential of the non-union sites continues to be mis-classified[20] and underestimated[21, 73]. The use of bone graft, most often autologous remains the standard in treatment of fracture non-union. It still has a high associated morbidity[64] and there is again a lack of clarity in the literature regarding the exact indications and timing of intervention. The work of Blick *et al*[105] remains the most widely quoted. Techniques and principles, particularly regarding soft tissue cover for severe fractures have evolved considerably[109] but from their heterogenous dataset they set the standard of two weeks following soft tissue cover

for autologous bone grafting that remains the practice today. Weber and Cech[16] reserved grafting mainly for large bone defects, particularly following infection and suggested that most often improving the stability is sufficient. Current indications are not clear and opinion therefore still relies on historical series. There have been no randomised controlled trials comparing grafting (including timings) and better mechanical stability alone. Instead, if anything a more aggressive approach that utilises revised fixation and orthobiologics[21, 73] (usually bone morphogenetic protein 7 (BMP7)) is being promoted by several groups. Other modalities of treatment are available but good evidence of their efficacy is lacking[110, 118-119, 122, 156-159].

6.4 Characterising the gap tissue in human fracture non-union

The gap tissue at the site of a fracture non-union is still considered to be either inert or inhibitory[21, 132]. This view has continually been challenged[16] and Iwakura *et al* [128] were able to isolate cells with osteoblastic potential from hypertrophic non-union tissue. Brownlow and coworkers[125-127] also suggested good biological potential in their work on animal models and human tissue. In the work presented in this thesis, a feasibility study was undertaken to determine if it were possible to quantify activity in the genes associated with bone healing processes from small samples of non-union tissue such that it could be related to the histological appearance and clinical behaviour. The hypothesis that there would be sufficient cells in sampled non-union tissue to profile activity in the genes related to fracture healing was supported. Uniformly only very small amounts of nucleotide was obtained from the sample. The tissues showed marked heterogeneity and when two samples from the same non-union were processed separately these also showed marked differences. The study did show that it was possible to profile the gene activity for osteoblastic and osteoclastic processes and supported the hypothesis. This was a feasibility study primarily due to time and financial constraints so further analyses was not possible. The implications are that the scintigraphy activity used by Weber and Cech[16] could be quantified as gene activity. The sample sizes were similar to those obtained for tissue biopsies of bone tumours meaning that it has potential clinical utility in determining healing potential and directing specific treatment modalities.

6.5 Matrix characteristics and fracture non-union

The histology presented here is illustrative of the tissue appearance. It was processed from tissue directly adjacent to that used for the RNA analysis and is therefore likely to share characteristics.

The matrix characteristics of the non-union tissue have not been given much consideration in the published literature but probably warrant further investigation. Ashhurst [139, 160]observed that osteoblastic replacement of the cartilage anlage was preceded by osteoclasts. Wassen *et al*[161] also commented on this and showed that there were differences in the collagen structure of the initial callus and the final collagen pattern of the remodelled bone. They demonstrated that the initial collagen, although Type I was subtly different to that of bone and promoted crystallisation between rather than within the helices. This fits with the descriptions by Wen *et al* [136] and Lawton *et al* [137-138] . The Wassen group then showed that the whole structure including the collagen I structure was then remodelled to achieve final healing. It is therefore feasible that non-union represents an arrest in the replacement of this initial matrix and is by definition therefore heterogenous the process stops or slows at different stages in different parts of the tissue and in different fractures. The matrix constituents are biologically active and binding sites differ depending on conditions. Surprisingly, there is virtually no work looking at the matrix morphology of non-union tissue and the osteoclast processes. In particular, further work has been proposed to determine if it is a lack of osteoclastic (brown on the TRAP staining here) binding moieties in the non-ossified collagenous matrix (blue on the Masson-Trichrome stains) prevents the active cellular areas from linking. If this were the case, then driving the biology alone is likely to fail unless it is preceded by a process that disrupts that block.

6.6 Animal models in fracture non-union

This work has again questioned the use of models to look at fracture non-union. Many gap models suggested that the biological agents used here had excellent potential[76-77, 79-80, 88-89, 99, 101, 104, 154-155] but they made no clinically significant impact on the model used here, one that is a closer representation of the clinically presenting non-union. Even this model has limitations and although models remain useful in refining research questions, they do not necessarily provide data that is open to direct clinical translation. Attempts to do so have led to relatively disappointing translational results[75, 110].

Further work is needed in properly characterising the non-union tissue itself, tissue that has too often been ignored in experimental design[132, 137-138]. In addition, particularly if models are to be used, the gap tissue from these needs to be compared to that from humans to validate the similarities and differences. The data presented here and in other work[126-128, 151] suggest that biological intervention may still have a role but the data from the animal model in this thesis emphasises that this needs to be better directed if translational strategies are to be

effective and reliable. This work demonstrated the feasibility of profiling the mesenchymal and osteoclastic activity of the gap tissue using very small samples. Work is ongoing using a large patient series to determine if the gene activities can be used to indicate healing potential along the lines of the trends demonstrated here. Fracture non-unions in patients are heterogenous at multiple levels and this also needs to be accounted for in experimental designs. The metabolic profile may be a useful tool in this regard.

6.7 Orthobiologics and fracture non-union

Orthobiological agents have shown potential particularly in gap models of non-union. This work used the same techniques and constructs in a rat model that has been validated and is analogous to the clinical situation. These agents were not sufficient when used in isolation in this model. The hypothesis for this work was that stimulation of the inherent potential of fracture non-union tissue with closed percutaneous injection of biologically active agents can lead to osseous union.

This work was unable to demonstrate evidence to support this hypothesis. The strength of the design is that it replicated the techniques and used the same constructs that have been successful in other models. The major difference is that it was delivered at a much later time point to replicate the clinical picture. The weaknesses are that the tissue has not been examined for markers of successful delivery nor the effective dose quantified. This needs to be addressed in future work before it can be said with certainty that the lack of effect is due to the chronic characteristics of the non-union rather than a methodological problem. It appears that stimulation of the tissue with orthobiological agents alone is insufficient. The recombinant protein technology, particularly when coupled with viral vectors as a delivery mechanism, appear to be very potent tools from laboratory data. However, until the biology of clinically presenting non-unions is better defined their translational impact may remain muted.

6.8 Further work

Fracture non-union presents many problems in terms of research as well as clinically. There is no real consensus with regard to definition and classification. Although the Weber and Cech system remains the most used it is widely misquoted making interpretation of results problematic. On a clinical level parameters need to be agreed with regard to reporting if data are to be appropriately compared. Only then can treatments from revision fixations and bone grafting through to the use of recombinant proteins be rationalised.

Non-union tissue has often been compared to healing or cancellous bone[127, 132]. The work of Weber and Cech[16], although over 35 years old remains very relevant particularly since they demonstrated that mechanical stability alone would lead to healing with most non-unions. One question raised by this thesis is: how do the gene profile and the histological appearance of the tissue change as it transforms from steady state non-union tissue to reactive tissue to bone? A second question is: can the changes induced by alteration of the forces be reproduced using a biological stimulus? Finally, the preliminary examination of the histology, in combination with the Ashhurst[139, 160] and Wassen[161] work question the role of the hitherto ignored osteoclasts. Frost[162] placed them at the front of the basic multicellular unit and they precede ossification. Therefore their histomorphometric distribution may be a crucial key to understanding why biological agents alone may be insufficient to achieve healing. Further work is therefore needed to elucidate the distribution of osteoclasts in non-union tissue and their relationship to the non-calcified matrix.

6.9 Concluding remarks

The hypothesis for the thesis was: there is quantifiable biological potential in fracture non-union tissue that can be stimulated leading to osseous union by closed percutaneous injection of induction factors. The work did not produce evidence to support that the biological potential of the non-union tissue can be stimulated to lead to healing by the percutaneous means described. It did however demonstrate the feasibility of quantifying the biological potential of the non-union gap tissue. It also demonstrated that it would be possible to correlate this to the histological appearance and clinical activity. These data have huge potential both in research and clinically.

The tissue at the site of a fracture non-union retains quantifiable metabolic activity. Although biological interventions have shown potential, they failed to produce healing in a more challenging clinically analogous model. Much remains to be understood about non-union tissue and the metabolic profiling demonstrated here is a potentially useful tool both experimentally and clinically. Further work is needed to refine and validate the assays used and to examine the architectural make up of the non-union tissue and the osteoclast activity patterns within this.

References

1. Johansen A, Evans RJ, Stone MD, Richmond PW, Su Vui L, Woodhouse KW. Fracture incidence in England and Wales: a study based on the population of Cardiff. Injury;28:655-60.
2. Gaston MS, Simpson AH. Inhibition of fracture healing. J Bone Joint Surg Br 2007;89:1553-60.
3. Dahabreh Z, Dimitriou R, Giannoudis PV. Health economics: a cost analysis of treatment of persistent fracture non-unions using bone morphogenetic protein-7. Injury 2007;38:371-7.
4. Goulet JA, Senunas LE, DeSilva GL, Greenfield ML. Autogenous iliac crest bone graft. Complications and functional assessment. Clin Orthop Relat Res 1997:76-81.
5. Heckman Jd Fau - Sarasohn-Kahn J, Sarasohn-Kahn J. The economics of treating tibia fractures. The cost of delayed unions.
6. Friedlaender GE, Perry CR, Cole JD, et al. Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions. J Bone Joint Surg Am 2001;83-A Suppl 1:S151-8.
7. Zimmermann G, Wagner C, Schmeckenbecher K, Wentzensen A, Moghaddam A. Treatment of tibial shaft non-unions: bone morphogenetic proteins versus autologous bone graft. Injury 2009;40 Suppl 3:S50-3.
8. hitzrot. The causes and the treatment of non-union. Am J Surg 1926;1:50-4.
9. Corrales LA, Morshed S, Bhandari M, Miclau T, 3rd. Variability in the assessment of fracture-healing in orthopaedic trauma studies. J Bone Joint Surg Am 2008;90:1862-8.
10. Bhandari M, Guyatt GH, Swiontkowski MF, Tornetta P, 3rd, Sprague S, Schemitsch EH. A lack of consensus in the assessment of fracture healing among orthopaedic surgeons. J Orthop Trauma 2002;16:562-6.
11. Tzioupis C, Giannoudis PV. Prevalence of long-bone non-unions. Injury 2007;38 Suppl 2:S3-9.
12. Court-Brown C MM, Tornetta III P, ed. Trauma. 1st ed. Philadelphia: Lippincott Williams & Wilkins; 2006.
13. USFDA. Guidance document for the exemptions and pre-market approval applications for bone growth stimulator devices. In: Administration USFaD, ed. Rockville, MD; 1988.
14. Frolke JP, Patka P. Definition and classification of fracture non-unions. Injury 2007;38 Suppl 2:S19-22.
15. Hamilton SW, Baird KS. The treatment of established non-union of the proximal humerus using the Polarus locking intramedullary nail. Int J Shoulder Surg 2009;3:53-6.
16. Weber BG CO, ed. Pseudoarthrosis:Pathology, Biomechanics, Therapy, Results. Bern: Hans Huber; 1976.
17. McKee M, ed. Aseptic non-union in AO Principles of fracture management. Stuttgart/New York: Thieme; 2007.
18. Paley D, Catagni MA, Argnani F, Villa A, Benedetti GB, Cattaneo R. Ilizarov treatment of tibial nonunions with bone loss. Clin Orthop Relat Res 1989:146-65.
19. Megas P. Classification of non-union. Injury 2005;36 Suppl 4:S30-7.
20. Marino JT, Ziran BH. Use of solid and cancellous autologous bone graft for fractures and nonunions. Orthop Clin North Am 2010;41:15-26; table of contents.
21. Giannoudis PV, Einhorn TA, Marsh D. Fracture healing: the diamond concept. Injury 2007;38 Suppl 4:S3-6.
22. Atkins RM. Principles of management of septic non-union of fracture. Injury 2007;38 Suppl 2:S23-32.
23. Simon AM, O'Connor JP. Dose and time-dependent effects of cyclooxygenase-2 inhibition on fracture-healing. J Bone Joint Surg Am 2007;89:500-11.
24. Gerstenfeld LC, Al-Ghawas M, Alkhiary YM, et al. Selective and nonselective cyclooxygenase-2 inhibitors and experimental fracture-healing. Reversibility of effects after short-term treatment. J Bone Joint Surg Am 2007;89:114-25.

25. Gullihorn L, Karpman R, Lippiello L. Differential effects of nicotine and smoke condensate on bone cell metabolic activity. J Orthop Trauma 2005;19:17-22.
26. Gruber R, Koch H, Doll BA, Tegtmeier F, Einhorn TA, Hollinger JO. Fracture healing in the elderly patient. Exp Gerontol 2006;41:1080-93.
27. Robinson CM, Court-Brown CM, McQueen MM, Wakefield AE. Estimating the risk of nonunion following nonoperative treatment of a clavicular fracture. J Bone Joint Surg Am 2004;86-A:1359-65.
28. Parker MJ. Prediction of fracture union after internal fixation of intracapsular femoral neck fractures. Injury 1994;25 Suppl 2:B3-6.
29. Brinker MR, Bailey DE, Jr. Fracture healing in tibia fractures with an associated vascular injury. J Trauma 1997;42:11-9.
30. Axelrad TW, Einhorn TA. Bone morphogenetic proteins in orthopaedic surgery. Cytokine Growth Factor Rev 2009;20:481-8.
31. Einhorn TA, Levine B, Michel P. Nutrition and bone. Orthop Clin North Am 1990;21:43-50.
32. Heppenstall RB, Brighton CT. Fracture healing in the presence of anemia. Clin Orthop Relat Res 1977:253-8.
33. Rothman RH, Klemek JS, Toton JJ. The effect of iron deficiency anemia on fracture healing. Clin Orthop Relat Res 1971;77:276-83.
34. Cozen L. Does diabetes delay fracture healing? Clin Orthop Relat Res 1972;82:134-40.
35. Gandhi A, Liporace F, Azad V, Mattie J, Lin SS. Diabetic fracture healing. Foot Ankle Clin 2006;11:805-24.
36. Loder RT. The influence of diabetes mellitus on the healing of closed fractures. Clin Orthop Relat Res 1988:210-6.
37. Brinker MR, O'Connor DP, Monla YT, Earthman TP. Metabolic and endocrine abnormalities in patients with nonunions. J Orthop Trauma 2007;21:557-70.
38. Ohlsson C, Bengtsson BA, Isaksson OG, Andreassen TT, Slootweg MC. Growth hormone and bone. Endocr Rev 1998;19:55-79.
39. Olney RC. Regulation of bone mass by growth hormone. Med Pediatr Oncol 2003;41:228-34.
40. Gerstenfeld LC, Einhorn TA. COX inhibitors and their effects on bone healing. Expert Opin Drug Saf 2004;3:131-6.
41. Gerstenfeld LC, Thiede M, Seibert K, et al. Differential inhibition of fracture healing by non-selective and cyclooxygenase-2 selective non-steroidal anti-inflammatory drugs. J Orthop Res 2003;21:670-5.
42. Giannoudis PV, MacDonald DA, Matthews SJ, Smith RM, Furlong AJ, De Boer P. Nonunion of the femoral diaphysis. The influence of reaming and non-steroidal anti-inflammatory drugs. J Bone Joint Surg Br 2000;82:655-8.
43. Waters RV, Gamradt SC, Asnis P, et al. Systemic corticosteroids inhibit bone healing in a rabbit ulnar osteotomy model. Acta Orthop Scand 2000;71:316-21.
44. Goodman SB, Jiranek W, Petrow E, Yasko AW. The effects of medications on bone. J Am Acad Orthop Surg 2007;15:450-60.
45. Kagel EM, Majeska RJ, Einhorn TA. Effects of diabetes and steroids on fracture healing. Curr Opin Orthop 1995;6:7-13.
46. Adams CI, Keating JF, Court-Brown CM. Cigarette smoking and open tibial fractures. Injury 2001;32:61-5.
47. Castillo RC, Bosse MJ, MacKenzie EJ, Patterson BM. Impact of smoking on fracture healing and risk of complications in limb-threatening open tibia fractures. J Orthop Trauma 2005;19:151-7.
48. Nasell H, Adami J, Samnegard E, Tonnesen H, Ponzer S. Effect of smoking cessation intervention on results of acute fracture surgery: a randomized controlled trial. J Bone Joint Surg Am 2010;92:1335-42.

49. Calori GM, Phillips M, Jeetle S, Tagliabue L, Giannoudis PV. Classification of non-union: need for a new scoring system? Injury 2008;39 Suppl 2:S59-63.
50. Abumunaser LA, Al-Sayyad MJ. Evaluation of the calori et Al nonunion scoring system in a retrospective case series. Orthopedics 2011;34:359.
51. Bhandari M, Tornetta P, 3rd, Sprague S, et al. Predictors of reoperation following operative management of fractures of the tibial shaft. J Orthop Trauma 2003;17:353-61.
52. MacKenzie EJ, Bosse MJ, Pollak AN, et al. Long-term persistence of disability following severe lower-limb trauma. Results of a seven-year follow-up. J Bone Joint Surg Am 2005;87:1801-9.
53. O'Toole RV, Castillo RC, Pollak AN, MacKenzie EJ, Bosse MJ. Determinants of patient satisfaction after severe lower-extremity injuries. J Bone Joint Surg Am 2008;90:1206-11.
54. Hammer RR, Hammerby S, Lindholm B. Accuracy of radiologic assessment of tibial shaft fracture union in humans. Clin Orthop Relat Res 1985:233-8.
55. Cunningham JL, Richardson JB, Soriano RM, Kenwright J. A mechanical assessment of applied compression and healing in knee arthrodesis. Clin Orthop Relat Res 1989:256-64.
56. Marsh D. Concepts of fracture union, delayed union, and nonunion. Clin Orthop Relat Res 1998:S22-30.
57. Esterhai JL, Jr., Brighton CT, Heppenstall RB, Alavi A, Mandell GA. Technetium and gallium scintigraphic evaluation of patients with long bone fracture nonunion. Orthop Clin North Am 1984;15:125-30.
58. Zimmermann G, Muller U, Wentzensen A. The value of laboratory and imaging studies in the evaluation of long-bone non-unions. Injury 2007;38 Suppl 2:S33-7.
59. Bhattacharyya T, Bouchard KA, Phadke A, Meigs JB, Kassarjian A, Salamipour H. The accuracy of computed tomography for the diagnosis of tibial nonunion. J Bone Joint Surg Am 2006;88:692-7.
60. Campbell RS, Grainger AJ, Hide IG, Papastefanou S, Greenough CG. Juvenile spondylolysis: a comparative analysis of CT, SPECT and MRI. Skeletal Radiol 2005;34:63-73.
61. Wiss DA, Stetson WB. Tibial Nonunion: Treatment Alternatives. J Am Acad Orthop Surg 1996;4:249-57.
62. Urist MR. Bone: formation by autoinduction. Science 1965;150:893-9.
63. Weber BG CO, ed. Autogenous grafting: techniques and indications. Bern: Hans Huber; 1976.
64. Banwart JC, Asher MA, Hassanein RS. Iliac crest bone graft harvest donor site morbidity. A statistical evaluation. Spine (Phila Pa 1976) 1995;20:1055-60.
65. Dahabreh Z, Calori GM, Kanakaris NK, Nikolaou VS, Giannoudis PV. A cost analysis of treatment of tibial fracture nonunion by bone grafting or bone morphogenetic protein-7. Int Orthop 2009;33:1407-14.
66. Lowe JA, Della Rocca GJ, Murtha Y, et al. Complications associated with negative pressure reaming for harvesting autologous bone graft: a case series. J Orthop Trauma 2010;24:46-52.
67. Porter RM, Liu F, Pilapil C, et al. Osteogenic potential of reamer irrigator aspirator (RIA) aspirate collected from patients undergoing hip arthroplasty. J Orthop Res 2009;27:42-9.
68. Urist MR, Silverman BF, Buring K, Dubuc FL, Rosenberg JM. The bone induction principle. Clin Orthop Relat Res 1967;53:243-83.
69. Urist MR, Strates BS. Bone morphogenetic protein. J Dent Res 1971;50:1392-406.
70. Termaat MF, Den Boer FC, Bakker FC, Patka P, Haarman HJ. Bone morphogenetic proteins. Development and clinical efficacy in the treatment of fractures and bone defects. J Bone Joint Surg Am 2005;87:1367-78.
71. Canalis E, Economides AN, Gazzerro E. Bone morphogenetic proteins, their antagonists, and the skeleton. Endocr Rev 2003;24:218-35.
72. Jones AL, Bucholz RW, Bosse MJ, et al. Recombinant human BMP-2 and allograft compared with autogenous bone graft for reconstruction of diaphyseal tibial fractures with cortical defects. A randomized, controlled trial. J Bone Joint Surg Am 2006;88:1431-41.

73. Calori GM, Colombo M, Ripamonti C, et al. Polytherapy in bone regeneration: clinical applications and preliminary considerations. Int J Immunopathol Pharmacol 2011;24:85-90.
74. Ronga M, Baldo F, Zappala G, Cherubino P. Recombinant human bone morphogenetic protein-7 for treatment of long bone non-union: an observational, retrospective, non-randomized study of 105 patients. Injury 2006;37 Suppl 3:S51-6.
75. Garrison KR, Shemilt I, Donell S, et al. Bone morphogenetic protein (BMP) for fracture healing in adults. Cochrane Database Syst Rev 2010;6:CD006950.
76. Amir LR, Li G, Schoenmaker T, Everts V, Bronckers AL. Effect of thrombin peptide 508 (TP508) on bone healing during distraction osteogenesis in rabbit tibia. Cell Tissue Res 2007;330:35-44.
77. Hanratty BM, Ryaby JT, Pan XH, Li G. Thrombin related peptide TP508 promoted fracture repair in a mouse high energy fracture model. J Orthop Surg Res 2009;4:1.
78. Hedberg EL, Tang A, Crowther RS, Carney DH, Mikos AG. Controlled release of an osteogenic peptide from injectable biodegradable polymeric composites. J Control Release 2002;84:137-50.
79. Li G, Cui Y, McIlmurray L, Allen WE, Wang H. rhBMP-2, rhVEGF(165), rhPTN and thrombin-related peptide, TP508 induce chemotaxis of human osteoblasts and microvascular endothelial cells. J Orthop Res 2005;23:680-5.
80. Sheller MR, Crowther RS, Kinney JH, et al. Repair of rabbit segmental defects with the thrombin peptide, TP508. J Orthop Res 2004;22:1094-9.
81. Wang Y, Wan C, Szoke G, Ryaby JT, Li G. Local injection of thrombin-related peptide (TP508) in PPF/PLGA microparticles-enhanced bone formation during distraction osteogenesis. J Orthop Res 2008;26:539-46.
82. Rodgers KE. Technology evaluation: Chrysalin (Orthologic). Curr Opin Mol Ther 2005;7:264-72.
83. Abraham LA, MacKie EJ. Modulation of osteoblast-like cell behavior by activation of protease-activated receptor-1. J Bone Miner Res 1999;14:1320-9.
84. Pagel CN, de Niese MR, Abraham LA, et al. Inhibition of osteoblast apoptosis by thrombin. Bone 2003;33:733-43.
85. Naldini A, Carraro F, Baldari CT, et al. The thrombin peptide, TP508, enhances cytokine release and activates signaling events. Peptides 2004;25:1917-26.
86. Norfleet AM, Huang Y, Sower LE, Redin WR, Fritz RR, Carney DH. Thrombin peptide TP508 accelerates closure of dermal excisions in animal tissue with surgically induced ischemia. Wound Repair Regen 2000;8:517-29.
87. Sower LE, Payne DA, Meyers R, Carney DH. Thrombin peptide, TP508, induces differential gene expression in fibroblasts through a nonproteolytic activation pathway. Exp Cell Res 1999;247:422-31.
88. Ryaby JT, Sheller MR, Levine BP, Bramlet DG, Ladd AL, Carney DH. Thrombin peptide TP508 stimulates cellular events leading to angiogenesis, revascularization, and repair of dermal and musculoskeletal tissues. J Bone Joint Surg Am 2006;88 Suppl 3:132-9.
89. Li G, Ryaby JT, Carney DH, Wang H. Bone formation is enhanced by thrombin-related peptide TP508 during distraction osteogenesis. J Orthop Res 2005;23:196-202.
90. Pountos I, Georgouli T, Henshaw K, Bird H, Jones E, Giannoudis PV. The effect of bone morphogenetic protein-2, bone morphogenetic protein-7, parathyroid hormone, and platelet-derived growth factor on the proliferation and osteogenic differentiation of mesenchymal stem cells derived from osteoporotic bone. J Orthop Trauma 2010;24:552-6.
91. Al-Zube L, Breitbart EA, O'Connor JP, et al. Recombinant human platelet-derived growth factor BB (rhPDGF-BB) and beta-tricalcium phosphate/collagen matrix enhance fracture healing in a diabetic rat model. J Orthop Res 2009;27:1074-81.

92. Groothuis A, Duda GN, Wilson CJ, et al. Mechanical stimulation of the pro-angiogenic capacity of human fracture haematoma: involvement of VEGF mechano-regulation. Bone 2010;47:438-44.
93. Hollinger JO, Hart CE, Hirsch SN, Lynch S, Friedlaender GE. Recombinant human platelet-derived growth factor: biology and clinical applications. J Bone Joint Surg Am 2008;90 Suppl 1:48-54.
94. Moore DC, Ehrlich MG, McAllister SC, et al. Recombinant human platelet-derived growth factor-BB augmentation of new-bone formation in a rat model of distraction osteogenesis. J Bone Joint Surg Am 2009;91:1973-84.
95. Tokunaga A, Oya T, Ishii Y, et al. PDGF receptor beta is a potent regulator of mesenchymal stromal cell function. J Bone Miner Res 2008;23:1519-28.
96. Govender S, Csimma C, Genant HK, et al. Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: a prospective, controlled, randomized study of four hundred and fifty patients. J Bone Joint Surg Am 2002;84-A:2123-34.
97. Hedberg EL, Kroese-Deutman HC, Shih CK, et al. Effect of varied release kinetics of the osteogenic thrombin peptide TP508 from biodegradable, polymeric scaffolds on bone formation in vivo. J Biomed Mater Res A 2005;72:343-53.
98. Evans CH, Ghivizzani SC, Robbins PD. Orthopedic gene therapy in 2008. Mol Ther 2009;17:231-44.
99. Betz VM, Betz OB, Glatt V, et al. Healing of segmental bone defects by direct percutaneous gene delivery: effect of vector dose. Hum Gene Ther 2007;18:907-15.
100. Virk MS, Conduah A, Park SH, et al. Influence of short-term adenoviral vector and prolonged lentiviral vector mediated bone morphogenetic protein-2 expression on the quality of bone repair in a rat femoral defect model. Bone 2008;42:921-31.
101. Betz OB, Betz VM, Nazarian A, et al. Direct percutaneous gene delivery to enhance healing of segmental bone defects. J Bone Joint Surg Am 2006;88:355-65.
102. Evans CH, Ghivizzani SC, Robbins PD. Progress and Prospects: genetic treatments for disorders of bones and joints. Gene Ther 2009;16:944-52.
103. Buckley RH. Gene therapy for SCID--a complication after remarkable progress. Lancet 2002;360:1185-6.
104. Baltzer AW, Lattermann C, Whalen JD, et al. Genetic enhancement of fracture repair: healing of an experimental segmental defect by adenoviral transfer of the BMP-2 gene. Gene Ther 2000;7:734-9.
105. Blick SS, Brumback RJ, Lakatos R, Poka A, Burgess AR. Early prophylactic bone grafting of high-energy tibial fractures. Clin Orthop Relat Res 1989:21-41.
106. Boyd HB, Lipinski SW, Wiley JH. Observations on Non-Union of the Shafts of the Long Bones, with a Statistical Analysis of 842 Patients. The Journal of Bone and Joint Surgery (American) 1961;43:159-68.
107. Souter WA. Autogenous cancellous strip grafts in the treatment of delayed union of long bone fractures. J Bone Joint Surg Br 1969;51:63-75.
108. Holden CE. Bone-grafts in the treatment of delayed union of tibial shaft fractures. Injury 1972;4:175-9.
109. Khan U, Kelly MB, Pleat J, Chesser TJ. Orthoplastics: An integral evolution within comprehensive trauma care. Injury 2011.
110. Aro HT, Govender S, Patel AD, et al. Recombinant human bone morphogenetic protein-2: a randomized trial in open tibial fractures treated with reamed nail fixation. J Bone Joint Surg Am 2011;93:801-8.
111. Physick PS. A case of fracture of the os humeri, in which the broken ends of the bone not uniting in the usual manner, a cure was effected by means of a seton. 1803. Clin Orthop Relat Res 2007;458:4-5.

112. Busse JW, Kaur J, Mollon B, et al. Low intensity pulsed ultrasonography for fractures: systematic review of randomised controlled trials. BMJ 2009;338:b351.
113. Ricardo M. The effect of ultrasound on the healing of muscle-pediculated bone graft in scaphoid non-union. Int Orthop 2006;30:123-7.
114. Khan Y, Laurencin CT. Fracture repair with ultrasound: clinical and cell-based evaluation. J Bone Joint Surg Am 2008;90 Suppl 1:138-44.
115. Jingushi S, Mizuno K, Matsushita T, Itoman M. Low-intensity pulsed ultrasound treatment for postoperative delayed union or nonunion of long bone fractures. J Orthop Sci 2007;12:35-41.
116. Simonis RB, Good C, Cowell TK. The treatment of non-union by pulsed electromagnetic fields combined with a Denham external fixator. Injury 1984;15:255-60.
117. Simonis RB, Parnell EJ, Ray PS, Peacock JL. Electrical treatment of tibial non-union: a prospective, randomised, double-blind trial. Injury 2003;34:357-62.
118. Scott G, King JB. A prospective, double-blind trial of electrical capacitive coupling in the treatment of non-union of long bones. J Bone Joint Surg Am 1994;76:820-6.
119. Bennett MH, Stanford R, Turner R. Hyperbaric oxygen therapy for promoting fracture healing and treating fracture non-union. Cochrane Database Syst Rev 2005:CD004712.
120. Hernigou P, Beaujean F. Abnormalities in the bone marrow of the iliac crest in patients who have osteonecrosis secondary to corticosteroid therapy or alcohol abuse. J Bone Joint Surg Am 1997;79:1047-53.
121. Hernigou P, Beaujean F. [Bone marrow in patients with pseudarthrosis. A study of progenitor cells by in vitro cloning]. Rev Chir Orthop Reparatrice Appar Mot 1997;83:33-40.
122. Hernigou P, Mathieu G, Poignard A, Manicom O, Beaujean F, Rouard H. Percutaneous autologous bone-marrow grafting for nonunions. Surgical technique. J Bone Joint Surg Am 2006;88 Suppl 1 Pt 2:322-7.
123. Hernigou P, Poignard A, Beaujean F, Rouard H. Percutaneous autologous bone-marrow grafting for nonunions. Influence of the number and concentration of progenitor cells. J Bone Joint Surg Am 2005;87:1430-7.
124. Calori GM, Tagliabue L, Gala L, d'Imporzano M, Peretti G, Albisetti W. Application of rhBMP-7 and platelet-rich plasma in the treatment of long bone non-unions: a prospective randomised clinical study on 120 patients. Injury 2008;39:1391-402.
125. Brownlow HC, Reed A, Simpson AH. The vascularity of atrophic non-unions. Injury 2002;33:145-50.
126. Brownlow HC, Reed A, Simpson AH. Growth factor expression during the development of atrophic non-union. Injury 2001;32:519-24.
127. Reed AA, Joyner CJ, Brownlow HC, Simpson AH. Human atrophic fracture non-unions are not avascular. J Orthop Res 2002;20:593-9.
128. Iwakura T, Miwa M, Sakai Y, et al. Human hypertrophic nonunion tissue contains mesenchymal progenitor cells with multilineage capacity in vitro. J Orthop Res 2009;27:208-15.
129. Seebach C, Henrich D, Tewksbury R, Wilhelm K, Marzi I. Number and proliferative capacity of human mesenchymal stem cells are modulated positively in multiple trauma patients and negatively in atrophic nonunions. Calcif Tissue Int 2007;80:294-300.
130. Kim CH, Kim KH, Jacobs CR. Effects of high frequency loading on RANKL and OPG mRNA expression in ST-2 murine stromal cells. BMC Musculoskelet Disord 2009;10:109.
131. Kim CH, You L, Yellowley CE, Jacobs CR. Oscillatory fluid flow-induced shear stress decreases osteoclastogenesis through RANKL and OPG signaling. Bone 2006;39:1043-7.
132. Hofmann A, Ritz U, Hessmann MH, et al. Cell viability, osteoblast differentiation, and gene expression are altered in human osteoblasts from hypertrophic fracture non-unions. Bone 2008;42:894-906.
133. Schell H, Lienau J, Epari DR, et al. Osteoclastic activity begins early and increases over the course of bone healing. Bone 2006;38:547-54.

134. Gerstenfeld LC, Alkhiary YM, Krall EA, et al. Three-dimensional reconstruction of fracture callus morphogenesis. J Histochem Cytochem 2006;54:1215-28.
135. Roach HI, Shearer JR, Archer C. The choice of an experimental model. A guide for research workers. J Bone Joint Surg Br 1989;71:549-53.
136. Wen HB, Cui FZ, Zhu XD. Microstructural features of non-union of human humeral shaft fracture. J Struct Biol 1997;119:239-46.
137. Lawton DM, Andrew JG, Marsh DR, Hoyland JA, Freemont AJ. Expression of the gene encoding the matrix gla protein by mature osteoblasts in human fracture non-unions. Mol Pathol 1999;52:92-6.
138. Lawton DM, Andrew JG, Marsh DR, Hoyland JA, Freemont AJ. Mature osteoblasts in human non-union fractures express collagen type III. Mol Pathol 1997;50:194-7.
139. Ashhurst DE. Collagens synthesized by healing fractures. Clin Orthop Relat Res 1990:273-83.
140. von der Mark K, Kirsch T, Nerlich A, et al. Type X collagen synthesis in human osteoarthritic cartilage. Indication of chondrocyte hypertrophy. Arthritis Rheum 1992;35:806-11.
141. dos Santos Neto FL, Volpon JB. Experimental nonunion in dogs. Clin Orthop Relat Res 1984:260-71.
142. Schmitz JP, Hollinger JO. The critical size defect as an experimental model for craniomandibulofacial nonunions. Clin Orthop Relat Res 1986:299-308.
143. White TO, Clutton RE, Salter D, Swann D, Christie J, Robinson CM. The early response to major trauma and intramedullary nailing. J Bone Joint Surg Br 2006;88:823-7.
144. Geris L, Reed AA, Vander Sloten J, Simpson AH, Van Oosterwyck H. Occurrence and treatment of bone atrophic non-unions investigated by an integrative approach. PLoS Comput Biol 2010;6:e1000915.
145. Garcia P, Holstein JH, Maier S, et al. Development of a reliable non-union model in mice. J Surg Res 2008;147:84-91.
146. Holstein JH, Garcia P, Histing T, et al. Advances in the establishment of defined mouse models for the study of fracture healing and bone regeneration. J Orthop Trauma 2009;23:S31-8.
147. Kaspar K, Matziolis G, Strube P, et al. A new animal model for bone atrophic nonunion: fixation by external fixator. J Orthop Res 2008;26:1649-55.
148. Oetgen ME, Merrell GA, Troiano NW, Horowitz MC, Kacena MA. Development of a femoral non-union model in the mouse. Injury 2008;39:1119-26.
149. Reed AA, Joyner CJ, Isefuku S, Brownlow HC, Simpson AH. Vascularity in a new model of atrophic nonunion. J Bone Joint Surg Br 2003;85:604-10.
150. Piska M, Yang L, Reed M, Saleh M. Drilling efficiency and temperature elevation of three types of Kirschner-wire point. J Bone Joint Surg Br 2002;84:137-40.
151. Boyan BD, Caplan AI, Heckman JD, Lennon DP, Ehler W, Schwartz Z. Osteochondral progenitor cells in acute and chronic canine nonunions. J Orthop Res 1999;17:246-55.
152. Qu G, von Schroeder HP. The osteogenic potential of pseudoarthrosis tissue and bone from human scaphoid non-unions. J Hand Surg Eur Vol 2008;33:449-56.
153. Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. J Cell Biochem 2003;88:873-84.
154. Li X, Wang H, Touma E, et al. TP508 accelerates fracture repair by promoting cell growth over cell death. Biochem Biophys Res Commun 2007;364:187-93.
155. Egermann M, Baltzer AW, Adamaszek S, et al. Direct adenoviral transfer of bone morphogenetic protein-2 cDNA enhances fracture healing in osteoporotic sheep. Hum Gene Ther 2006;17:507-17.
156. Aaron RK, Ciombor DM, Simon BJ. Treatment of nonunions with electric and electromagnetic fields. Clin Orthop Relat Res 2004:21-9.

157. Bielecki T, Gazdzik TS, Szczepanski T. Benefit of percutaneous injection of autologous platelet-leukocyte-rich gel in patients with delayed union and nonunion. Eur Surg Res 2008;40:289-96.
158. Heckman Jd Fau - Ryaby JP, Ryaby Jp Fau - McCabe J, McCabe J Fau - Frey JJ, Frey Jj Fau - Kilcoyne RF, Kilcoyne RF. Acceleration of tibial fracture-healing by non-invasive, low-intensity pulsed ultrasound.
159. Karamitros AE, Kalentzos VN, Soucacos PN. Electric stimulation and hyperbaric oxygen therapy in the treatment of nonunions. Injury 2006;37 Suppl 1:S63-73.
160. Bland YS, Critchlow MA, Ashhurst DE. The expression of the fibrillar collagen genes during fracture healing: heterogeneity of the matrices and differentiation of the osteoprogenitor cells. Histochem J 1999;31:797-809.
161. Wassen MH, Lammens J, Tekoppele JM, et al. Collagen structure regulates fibril mineralization in osteogenesis as revealed by cross-link patterns in calcifying callus. J Bone Miner Res 2000;15:1776-85.
162. Frost HM. Bone "mass" and the "mechanostat": a proposal. Anat Rec 1987;219:1-9.